ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 95, ART. 2 PAGES 741–1020

THE USE OF ANIMAL CELL, TISSUE, AND ORGAN CULTURES IN RADIOBIOLOGY

Conference Editor

CHARLES M. POMERAT

LIST OF AUTHORS

CHARLES M. POMERAT AND DAVID C. WHITE (*Conference Cochairmen*), J. R. ANDREWS, A. AWA, R. BASES, R. J. BERRY, J. L. BIEDLER, E. BORGHESE, A. M. BRUES, J. G. CARLSON, A. R. T. DENUES, J. FOGH, G. O. GEY, M. K. GEY, A. GOLDFEDER, H. HARRINGTON, H. B. HEWITT, W. L. HUGHES, W. H. LANGHAM, I. LASNITZKI, W. B. LOONEY, T. R. MUNRO, Y. OHNUKI, E. E. OSGOOD, R. B. PAINTER, D. F. PETERSEN, D. E. ROUNDS, P. C. SANDERS, A. SCHLEICH, R. SCHREK, A. N. STROUD, B. R. SVOBODA, J. E. TILL, L. J. TOLMACH, O. A. TROWELL, AND C. W. WILSON

Editor

FRANKLIN N. FURNESS

Managing Editor

EDGAR W. WHITE

Associate Editor

MALCOLM EASTERLIN

NEW YORK

PUBLISHED BY THE ACADEMY

November 13, 1961

THE NEW YORK ACADEMY OF SCIENCES

(Founded in 1817)

BOARD OF TRUSTEES

BORIS PREGEL, *Chairman of the Board*

Class of 1960–1961

HARDEN F. TAYLOR

Class of 1960–1962

W. STUART THOMPSON

Class of 1960–1963

HENRY C. BRECK

LOWELL C. WADMOND

Class of 1961–1964

GORDON Y. BILLARD BORIS PREGEL

FREDERICK Y. WISELOGLE, *President of The Academy*

M. J. KOPAC, *Past President* HILARY KOPROWSKI, *Past President*

EUNICE THOMAS MINER, *Executive Director*

SCIENTIFIC COUNCIL, 1961

President, FREDERICK Y. WISELOGLE

President-Elect, JAMES B. ALLISON

EMERSON DAY, *Vice-President* CHARLES W. MUSHETT, *Vice-President*

Recording Secretary

KARL MARAMOROSCH

Corresponding Secretary

ROSS F. NIGRELLI

Elected Councilors

1959–1961

JOHN E. DEITRICK CHARLES W. MUSHETT

ROBERT S. MORISON E. L. TATUM

1960–1962

JOHN JOSEPH LYNCH, S.J. MORRIS SCHAEFFER

1961–1963

JACOB FELD ANDRES FERRARI

Executive Director, EUNICE THOMAS MINER

SECTION OF BIOLOGICAL AND MEDICAL SCIENCES

CHARLES R. NOBACK, *Chairman* PRESTON L. PERLMAN, *Vice-Chairman*

DIVISION OF ANTHROPOLOGY

ETHEL BOISSEVAIN, *Chairman* ROBERT HECKEL, *Vice-Chairman*

DIVISION OF INSTRUMENTATION

ANDRES FERRARI, *Chairman* WALTER E. TOLLES, *Vice-Chairman*

DIVISION OF MICROBIOLOGY

EMANUEL GRUNBERG, *Chairman* H. CHRISTINE REILLY, *Vice-Chairman*

DIVISION OF PSYCHOLOGY

LOUIS W. MAX, *Chairman* GEORGE K. BENNETT, *Vice-Chairman*

SECTION OF CHEMICAL SCIENCES

FREDERICK R. EIRICH, *Chairman* A. D. SHABICA, JR., *Vice-Chairman*

DIVISION OF BIOCHEMISTRY

RAYMOND L. GARNER, *Chairman* J. J. BURNS, *Vice-Chairman*

SECTION OF GEOLOGICAL SCIENCES

R. W. FAIRBRIDGE, *Chairman* BARTHOLOMEW NAGY, *Vice-Chairman*

DIVISION OF OCEANOGRAPHY AND METEOROLOGY

CHARLES KNUDSEN, *Chairman* JAMES K. McGUIRE, *Vice-Chairman*

SECTION OF PHYSICAL SCIENCES

HIRAM HART, *Chairman* ROBERT D. HATCHER, *Vice-Chairman*

DIVISION OF ENGINEERING

JACOB FELD, *Chairman* JOSEPH F. SKELLY, *Vice-Chairman*

DIVISION OF MATHEMATICS

BRADFORD F. HADNOT, *Chairman* MARY P. DOLCIANI, *Vice-Chairman*

Past Presidents

M. J. KOPAC HILARY KOPROWSKI

The Sections and the Divisions hold meetings regularly, one evening each month, during the academic year, October to May, inclusive. All meetings are held at the building of The New York Academy of Sciences, 2 East Sixty-third Street, New York 21, New York.

Conferences are also held at irregular intervals at times announced by special programs.

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 95, ART. 2 PAGES 741–1020

November 13, 1961

THE USE OF ANIMAL CELL, TISSUE, AND ORGAN CULTURES IN RADIOBIOLOGY*

CONTENTS

* This series of papers is the result of a conference entitled *The Use of Animal Cell, Tissue, and Organ Cultures in Radiobiology* held by The New York Academy of Sciences on February 16, 17, and 18, 1961.

GROWTH PATTERNS IN X-IRRADIATED HeLa CELLS*

L. J. Tolmach

The Edward Mallinckrodt Institute of Radiology, School of Medicine, and Committee on Molecular Biology, Washington University, St. Louis, Mo.

Effects of ionizing radiations on cells in culture have been studied for many years, and a number of observationally distinct responses have been recorded. Thus, in addition to the clearly genetic changes revealed as chromosome aberrations and mutations, alterations in growth patterns occur, both temporary and permanent, including immediate or delayed reproductive death, delay in mitosis, cellular enlargement to giant forms, reduced synthetic rates, fusion between cells, metabolic death, and necrosis. Study of these cellular changes, and of their causes and relationships, requires that the consequences of exposure to radiations be given precise description, but the multiplicity of responses tend to obscure the effect on any single growth parameter. In addition the obligation, until recently, that mass populations be employed had made it difficult to study responses of individual cells. With the development of techniques whereby single, isolated cells can be grown reliably (Puck *et al.*, 1956), it has become possible to make repeated observations of sizeable populations during the postirradiation period, and to document the individual responses of large numbers of cells.

Accordingly a study was initiated, using these techniques, directed toward delineation and accurate description of a few of the above-mentioned responses of the HeLa S3 cell strain to 220 kev. X rays (Tolmach *et al.*, 1960). In particular, measurement of the dose dependence of certain responses was undertaken with the aim of establishing correlations between these observable effects of irradiation.

Materials and Methods

Two strains of HeLa cells† were studied: S3-9 and its derivative S3-9IV (Marcus, 1959). No differences were observed in their responses to radiation. They were grown and manipulated in medium N16HHF and otherwise handled by standard procedures (Ham and Puck, 1961). Incubation was carried out at 37° C. in an atmosphere of 96.5 per cent air +3.5 per cent carbon dioxide.

Cells were trypsinized, disaggregated, diluted in a large volume of medium, and either maintained in suspension by continued stirring (suitable aliquots being removed and plated after measured exposures) or plated and, finally, irradiated. When it was necessary to make an observation at the time of irradiation, the seeded plates were incubated for about 3 hours prior to irradiation to allow the cells to attach to the growth surface.

About 10^3 cells were plated in 50 mm. glass or plastic Petri dishes. In order to make repeated observations of the same cells, aluminum masks bearing an array of holes of the same diameter as the microscopic field were fixed to the bottom of the Petri dishes (Marcus and Puck, 1958). Observations were made

* The work described in this paper was supported in part by Research Grant C-4483 from the National Cancer Institute, Public Health Service, Bethesda, Md.

† Generously provided by T. T. Puck and P. I. Marcus.

with inverted microscopes equipped with either 5× bright field or 10× phase-contrast objectives.

Other procedures used in certain of the experiments are described in the appropriate sections below.

Irradiations were performed at room temperature with a Westinghouse Quadrocondex unit operated at 220 kv. C.P., 15 mAmp., with 1 mm. Al + 0.5 mm. Cu added filtration. The half-value layer was 1.9 mm. Cu. Dose rates varied from 20 to 80 rpm. Relative doses are accurately recorded for each type of experiment, but correction has not been made for scatter from the glass dish, which results in a 40 per cent increase in dose (Hood and Norris, 1961) in a few experiments. The interpretation of the results is not affected. In general only the effects produced by exposure to several hundred roentgens or more were recorded, as it was desired to limit this study to reproductively dead cells.

Results and Discussion

Population Dynamics

After exposure to doses of X rays sufficient to cause appreciable reproductive death, cell populations must undergo marked departure from the normal exponential increase in number. The growth rates of the cultures may ultimately achieve normal values but, before they do so, delay in division, abortive reproduction, cell fusion, and disintegration of cells may combine to produce progressively lower minima in the growth curves, with increasing doses. At the times of minimum cell number, considerable magnification of the effects of irradiation may be achieved. While measurement of total cell numbers may therefore be a useful indicator of radiation damage, such data do not bear a simple relation to reproductive death, nor do they yield sufficiently detailed accounts of the events occurring during the postirradiation period. Hence alternative indexes of growth were examined. Measurements of mitotic activity had been carried out on irradiated-cell populations (Lasnitzki, 1943), and attempts were made to repeat these on HeLa cells. However, it has proved extremely difficult to obtain reproducible mitotic counts in irradiated populations. Furthermore, mitotic activity may be an erroneous index of cell multiplication, as the mitotic stage may be prolonged in irradiated cells, and may fail to be followed by cytokinesis.

Alternatively information about postirradiation growth has been derived from repeated observations of a large number of individual cells. These permit resolution of the over-all changes in cell number into some of the processes mentioned. Ideally the irradiated population should be monitored continuously since, if both multiplication and cell loss occur in a given microcolony during the interobservational period, neither will be detected. Practically, it has not proved feasible to make observations more frequently than at 6-hour intervals or, in some experiments, at 24-hour intervals. Such intermittent observation results in an underestimate of both cell loss and multiplication, as shown by data cited below under *Cell Proliferation*, which prove that an appreciable number of divisions and losses go undetected if observations are delayed to 24-hour as compared to 6-hour intervals. In addition, reliable detec-

tion of incomplete cytokinesis that may be followed by fusion is entirely precluded; only time-lapse cinemicrography can yield information about the latter process. At low doses, where the mitotic lag is short and most cells divide a number of times before the colony aborts, even 6-hour intervals between observations are too long to reveal cell loss accurately, at least during the first several days after irradiation. Consequently data are generally presented for doses above 800 r.

The criteria adopted for deciding whether an entity was one cell attached to the growth surface, or two, were of necessity somewhat arbitrary. Because a normal cell observed in mitosis invariably appeared as two cells at the next observation and thus was scored as two, a mitotic cell was counted as two in the irradiated populations also. However there is no assurance that two completely separate cells always developed in the latter; there were in fact occasional instances suggestive of sister-cell fusion. Furthermore some of the cells in mitosis were undoubtedly about to detach permanently from the growth surface when observed. Still, most cell counts did not involve uncertainties of this sort, as only relatively few mitotic events were observed. Of greater concern was the appreciable frequency of cytoplasmic bridges between cells; the sister cells were scored as two. For these reasons the criteria employed here yield a division frequency that is somewhere between the mitotic frequency and the frequency of completed cytokinesis, and is probably closer to the former.

FIGURE 1 shows crude growth curves for cells that have been irradiated with 0.8 or 4.0 kr. In order to resolve these data into curves for cell proliferation and cell disappearance, the histories of many hundreds of individual cells were recorded. Repeated observations of specified fields permitted separate scoring of multiplication and loss; such resolution of the curves of FIGURE 1 is depicted in FIGURE 2. The upper, solid curves represent cell increase via division, while the lower, dashed curves show loss of cells from the growth surface. The two processes will be discussed below.

Cell proliferation. The curves for cell increase were constructed by counting the number of new cells arising by division during the time between each successive pair of observations, and expressing this as the fractional increase in cell number. Much of the complexity of the unresolved growth curves is retained, and may be analyzed in terms of 3 parameters pertaining to cell multiplication: (1) growth rate, (2) division lag, and (3) the number of divisions that the cells complete.

(1) Because most cells undergo only a very few divisions after irradiation with these doses, the fraction that can be expected to divide is small at all times, at least after the first division. Hence the slopes of the curves do not yield meaningful measures of the growth rate. It is feasible, however, to determine whether the few divisions that occur do so at normal intervals, especially after the smaller exposures. In one experiment with 400 r, this appeared to be the case (TABLE 1), if it is assumed that all the cells destined to divide a second time had done so by the time observations were discontinued.

(2) The cumulative percentages of cells undergoing their first postirradiation division are plotted in FIGURE 3 as a function of time after receiving 3 different doses of X rays. Data from 2 separate experiments are included; the dis-

crepancy between the 2 control curves indicates the small but not negligible variability encountered among experiments. It is clear that while a small proportion of cells divides soon after irradiation, the vast majority is delayed about 1 min./r in the dose range 0.4 to 2.0 kr. This value is close to that reported by Whitfield and Rixon (1959) for mouse L cells irradiated with the

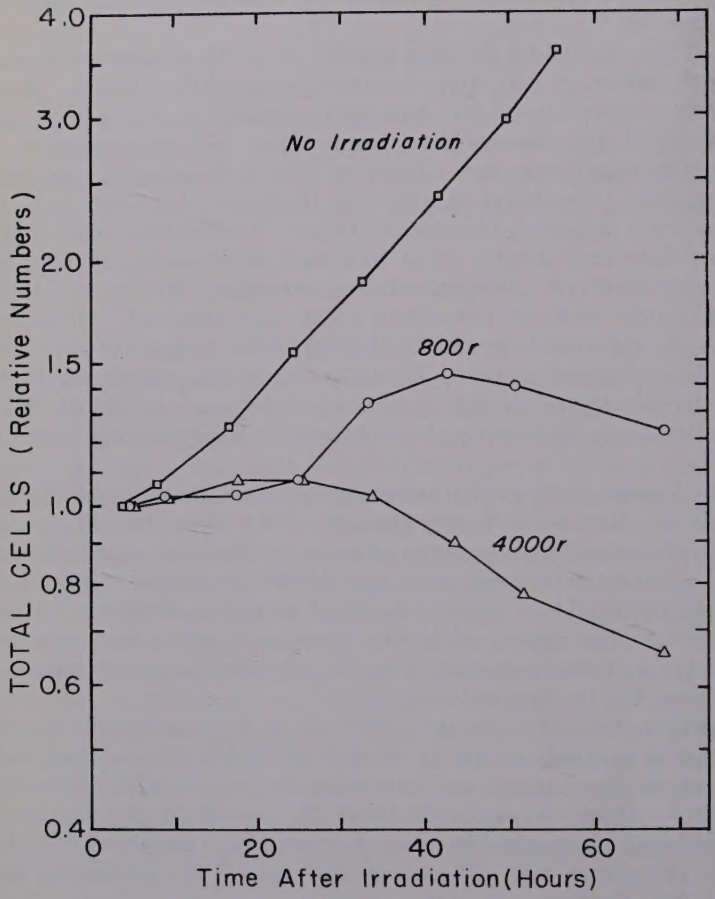


FIGURE 1. Over-all growth curves of HeLa S3-9 cells after doses of 800 and 4000 r of 220 kv. X rays. The total number of cells in specified microscopic fields is plotted for each observation. The number of cells at the first observation was 308, 346, and 228, respectively, for the control, 800 r, and 4000 r curves.

same doses. Although the curves of FIGURE 3 have been drawn to maximize the uniformity of division delay among the cells of the irradiated populations, it is not clear from the data that delay is in fact uniform. Indeed preliminary work with synchronized populations indicates that cells are more susceptible to temporary inhibition of division (as well as to several other measures of radiation damage) at certain times during the division cycle than at other times. The cause of lag remains unclear, although recent experiments of several

workers indicate that the premitotic phase of the division cycle is the period that is affected (Whitmore *et al.*, 1961).

(3) The data shown in FIGURE 3 also reveal that the fraction of cells undergoing even 1 division is severely reduced by the X-ray doses administered; none of the irradiated populations reach the control level of division. Puck

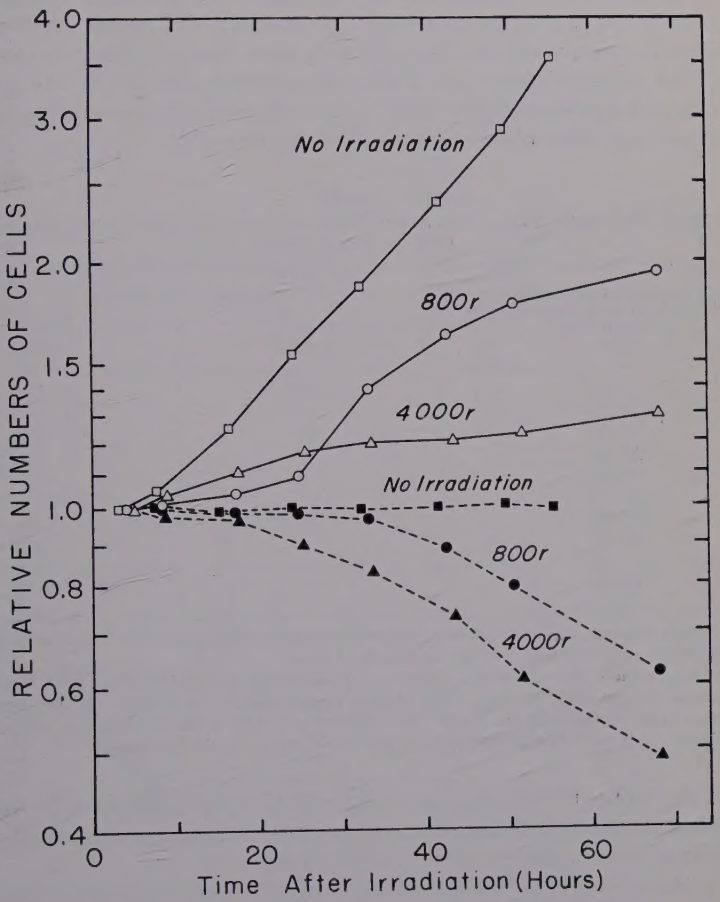


FIGURE 2. Resolution of the data of FIGURE 1 into cell increase via division (*upper set of solid curves*), and loss of cells from the glass surface (*lower set of dashed curves*).

and Marcus (1956) reported that the average size of abortive colonies 9 days after irradiation is markedly reduced by relatively small doses of X rays. Repeated intermittent observations now make it clear that cell division is indeed severely reduced. For example daily observation indicated that 50 per cent of the cells failed to divide once, after receiving doses greater than about 500 r (FIGURE 4). However, quantitation of this effect is unreliable unless cells are monitored very frequently. Thus in one experiment observations at 24-hour intervals indicated that only 52 of 99 cells divided after exposure to 800 r,

while observations at 6-hour intervals yielded a count of 86 dividers. These observations showed also that a limiting inhibition of cell division is reached at a few thousand roentgens. However, again, the fraction of cells scored as dividing at this limit is about 10-fold greater with 6-hour observation (FIGURE 5) than with daily observations. These discrepancies arise from failure to observe those cells that are lost through metabolic death or that undergo fusion during the interobservational period. Presumably still higher levels of post-irradiation division would be detected with more frequent observations.

The data obtained from the 6-hour observations do not fit the empirical equations of Engelberg (1960), which were derived partly from experiments at lower doses, and from observations at 12-hour intervals.

TABLE 1

INTERVAL BETWEEN FIRST AND SECOND DIVISIONS IN CONTROL AND IRRADIATED (400 r) POPULATIONS

Maximum interval between appearance of 2nd cell and 3rd (or 3rd and 4th) cell in microcolony (hrs.)	Number of microcolonies	
	Control	400 r
5–10	4*	1*
10–15	2*	1*
15–20	0	3*
20–25	8	5
25–30	4	2
30–35	17	14
35–40	23†	12
40–45	3	0
Totals	61	38

* The high frequency of rapid second divisions suggests that growth of these cells may have been somewhat perturbed as a result of manipulation.

† The long modal time does not reflect a slow-growing population, but long intervals between certain of the observations, which lead to large maximum times. While it is possible that the actual interdivisional times were shorter for the control than for the irradiated populations, this is considered unlikely in view of the similar distributions.

As in the case of mitotic lag, loss of divisional capacity may not be randomly distributed in the population but, rather, may be more pronounced in cells irradiated at certain points in the mitotic cycle.

It is postulated that loss of the ability to divide after irradiation is an alternative indicator of the lesion which leads to reproductive death. That is, although the dose dependencies of these two indexes of radiation damage are not commensurable, the rapid drop in divisional capacity with increasing dosage may be attributed to early reproductive death. This interpretation is consistent with the relatively high plateau in dose response represented by FIGURE 5; since these cells remain intact for at least 1 day after doses of even 5 kr (see under *Loss of Cells* below), and are actively metabolizing during this time (Tolmach and Marcus, 1960), they may well be able to attempt 1 division. Disruption of their genetic apparatus consequent to that divisional attempt, however, may prevent their dividing further. Indeed it may lead to their immediate disintegration.

Loss of cells. The loss of irradiated HeLa S3 cells from the growth surface of the culture vessel during incubation after exposure to 1.3 kr was described previously (Tolmach and Marcus, 1960). This radiation effect has been fur-

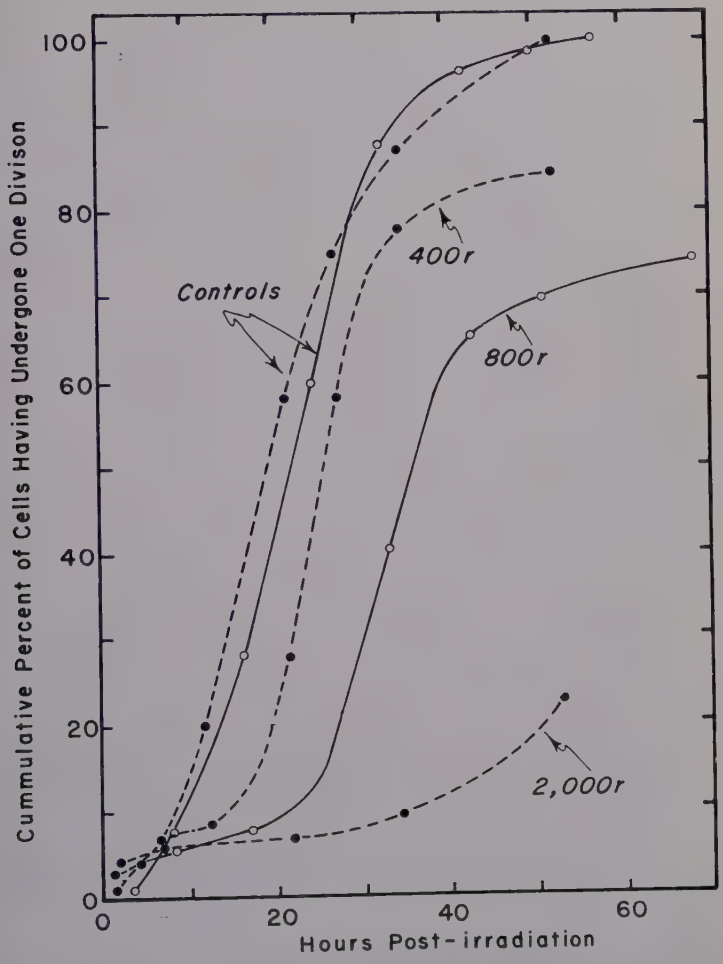


FIGURE 3. Cumulatative percentages of cells undergoing the first postirradiation division. Data from 2 experiments, both involving more than 200 cells, are shown. The control curves have been normalized to 100 per cent division at 52 to 57 hours. Actual values were 91.5 per cent (*dashed curve*), and 98.5 per cent (*solid curve*). The actual values probably closely represent the plating efficiencies. The control-curve data indicate that several hours of lag were introduced by the manipulation at the time of irradiation.

ther studied during a 2-year period, mostly in long-term experiments in which observations were made daily for a period as long as 3 weeks but also in shorter experiments in which cells were observed more frequently. Although data obtained by making observations only once a day tend to underestimate slightly cell loss during the first few days after irradiation, the over-all curves derived

from the data may be taken as valid representations of the loss of irradiated cells.

Results of the short-term experiments are typified by the dashed curves of FIGURE 2, which were constructed by scoring the cells lost between two observations, calculating the fraction of those recorded at a given observation which were still present at the next observation, and normalizing to the number present at the beginning of the experiment. It is seen that cell loss does not commence until about the second day postirradiation, the exact time depending

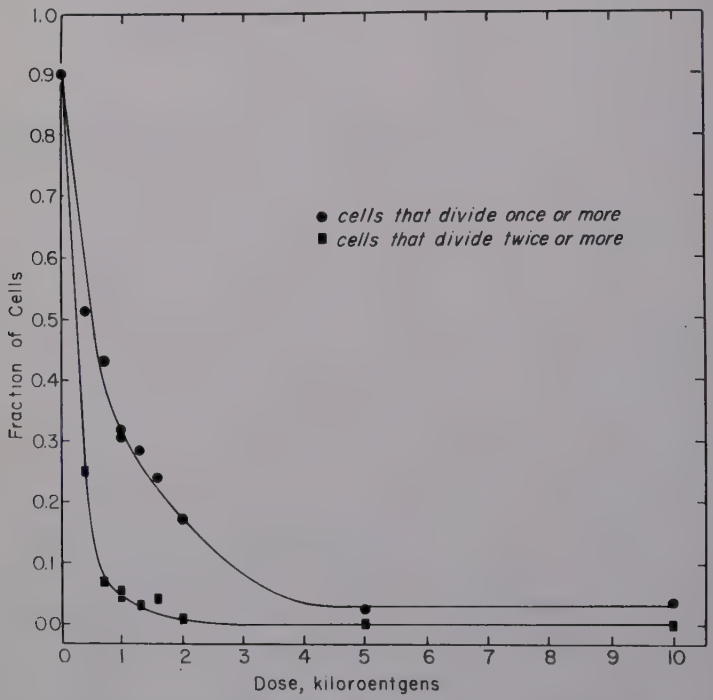


FIGURE 4. Dose dependence of cell division. The fraction of cells dividing once or more (*upper curve*), and twice or more (*lower curve*), as determined from daily observations, is plotted as a function of dose.

inversely on the dose. Long-term persistence curves typical of those obtained during one 6-mo. period are shown in FIGURE 6. Several features of these experiments may be noted:

(1) The shapes of the curves and the parameters characterizing them differ from those presented earlier (Tolmach and Marcus, 1960). In addition, more recently obtained curves, although still of the same general shape, are slightly steeper. These changes occurring over long periods possibly are reflections of small alterations of general cultural conditions, or of the cell strain. Irradiated cells appear to be sensitive indicators of certain types of alterations in the environment; for example, they are more susceptible than normal cells to cytopathic response on infection with some viruses (Cieciura *et al.*, 1957; Levine,

1960). However no correlation has been observed between general growth conditions, as revealed by plating efficiency or growth rate of normal cells, and persistence of irradiated cells.

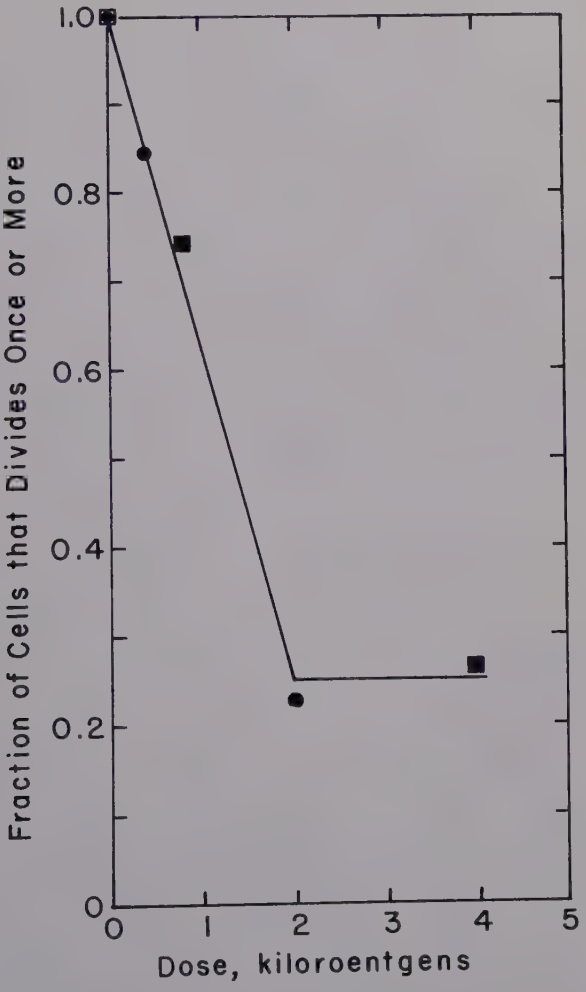


FIGURE 5. Dose dependence of cell division. The fraction of cells dividing once or more, as determined from observations at 6-hour intervals, is plotted against dose. Data are from 2 experiments.

(2) There is an extremely broad spread of persistence times in the irradiated population. In this series of experiments the population appears to consist of at least 2 fractions, the first (70 to 80 per cent) being lost relatively rapidly during the 2nd to 5th day following irradiation, but the 2nd (20 to 30 per cent) being lost exponentially with a 1/e time of about 160 hours.

(3) The rapidly lost fraction shows a small dose dependence. At the higher

doses tested (4 to 10 kr), loss commences somewhat sooner than at 1 to 2 kr (FIGURES 2 and 6).

(4) Both the size of the slow fraction and its rate of detachment are independent of dose in the range 1 to 10 kr (TABLE 2).

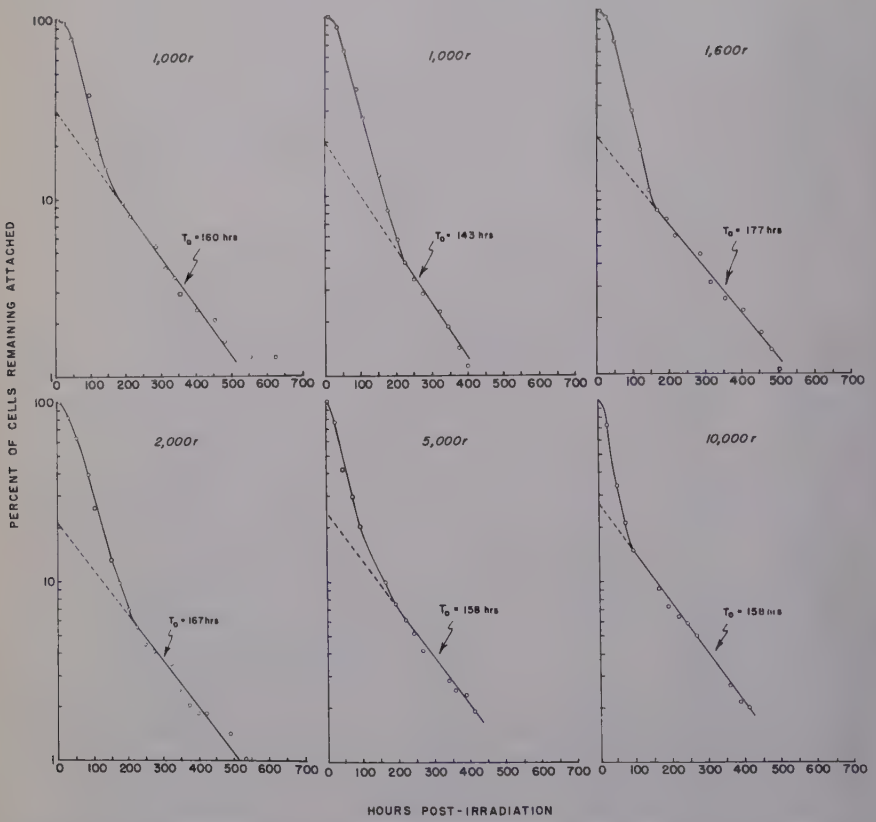


FIGURE 6. Persistence of irradiated cells on the growth surface after exposure to different doses of X rays. Observations were made at daily intervals. The data have been adjusted to take into account the small amount of cell division that was observed at early times. The 2 curves for 1000 r indicate the magnitude of variation obtained during one 6-mo. period. T_0 refers to the 37 per cent persistence time for the terminal slope. The initial number of cells under observation in the experiments of the upper row of curves was (reading from left to right) 276, 431, and 377 and, of the lower row, 357, 677, and 600.

(5) No difference in persistence could be detected between cells that divided (daily observations) and those that did not (FIGURE 7).

(6) After trypsinization on the 6th, 13th, or 15th day following irradiation, two thirds to three quarters of the persistent cells reattach to the glass surface (TABLE 3). The further persistence of these cells was not altered by their having undergone trypsinization.

(7) A second comparable dose of X rays two days after the first did not alter persistence.

TABLE 2

PERSISTENCE OF THE FRACTION OF THE IRRADIATED POPULATION THAT DETACHES SLOWLY

Dose (kr)	Fraction of cells lost slowly (extrapolated to time of irradiation)	Time for 37 per cent persistence (T_0) (hrs.)
1.0	0.31	160
1.0	0.20	143
1.3	0.23	163
1.6	0.20	177
2.0	0.21	167
5.0	0.26	158
10.0	0.27	158

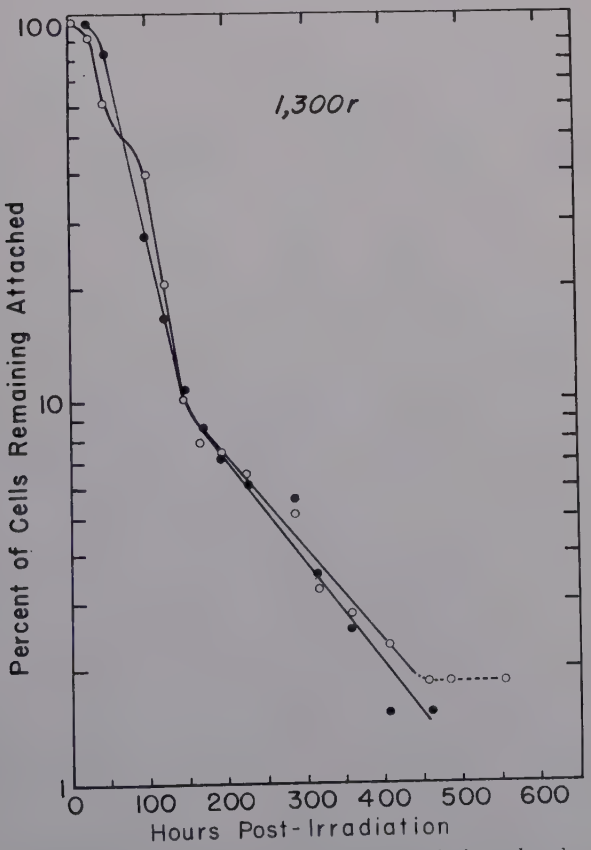


FIGURE 7. Persistence of cells that divide as compared with those that do not, as revealed by daily observations after exposure to 1.3 kr. *Solid circles*, 83 dividers; *open circles*, 215 nondividers. The 2 curves do not significantly differ.

In previous work it was shown that the persistent cells of HeLa and other strains continue to enlarge during at least a large portion of the postirradiation period and, therefore, are metabolically active (see under *Growth of Individual Cells* below). Other indications of metabolic activity that have been cited (Tolmach and Marcus, 1960), and the present experiments on reattachment of trypsinized cells (TABLE 3) add support to the conclusion that persistent cells are physiologically active. Conversely the cells that detach from the growth surface do not reattach, and they undergo morphological disintegration rapidly. Hence we may tentatively take cell detachment as an indicator of metabolic death, and may inquire into the causes of such death.

The results cited above suggest that metabolic death is an event physiologically remote from the immediate results of the radiation insult. Thus it is relatively dose-independent (FIGURE 6), and it is equally likely to occur in a cell that has divided as in one that has not (FIGURE 7), although division is very sensitive to dose. One explanation for this physiological isolation derives from the relative insensitivity of over-all metabolism to radiation insult, as

TABLE 3
REATTACHMENT AND PERSISTENCE OF IRRADIATED CELLS (2000 r) AFTER SECOND TRYPSINIZATION

Time after irradiation at which population was trypsinized (hrs.)	Fraction of cells remaining at time of trypsinization	Minimum* fraction of trypsinized cells that reattach	Time for 37 per cent persistence (T_0)† (hrs.)
136	0.174	0.75	140
324	0.020	0.68	175
368	0.015	0.68	175

* The measured values are minima, as immediate scoring is impossible, a few hours being required for reattachment.

† T_0 is given for the majority fraction of cells if two slopes were apparent.

mentioned above and below under *Growth of Individual Cells*, and from early findings that cellular degeneration following irradiation occurred concomitantly with the reappearance of mitotic activity after the radiation-induced lag. From this it was concluded that cells die metabolically as a consequence of attempted mitosis (see Lea, 1956, chapter IX). A test for the presence of such a mechanism in this system has not yet been made. However, the condition of the nucleus in loosely attached cells was examined during the rapid phase (2 to 4 days after irradiation) of detachment. TABLE 4 shows that while one quarter to one half of the cells collected show mitoses that may reflect normal divisional activity, the nuclei of about one half the cells are abnormal. In contrast only about 10 per cent of cells collected from normal populations show pycnotic nuclei. It thus appears that nuclear degeneration is an early manifestation of metabolic death. Such degeneration might follow mitotic activity. By suitable scheduling of collections of detached cells, it should be possible to investigate further the relation of mitosis to this process, and the sequence of events at this critical time in the irradiated cell's existence.

However it remains to be established whether any mitotic activity at all can be detected when cells that persist for many days finally detach. The

absence of mitoses in irradiated populations reported by Puck and Marcus (1956) suggests that some other type of process may be occurring that leads to cell death. In addition, the manner in which to reconcile, in terms of mitotic death, the slightly decreased persistence time of the majority of the population at high doses with the observation that postirradiation mitotic lag is increased with dose, is not apparent.

While it is clear that, under the conditions of these experiments, failure of cells to persist after 5 days is independent of dose and that, therefore, differences in persistence time cannot be ascribed to variations in the amount of radiation damage inflicted, it is possible that the cells of a randomly growing population are subject to different responses, depending on their position in the division cycle. Experiments with synchronized population should resolve the question. However, even if such differences are found, it is hardly to be expected that

TABLE 4
CONDITION OF THE NUCLEUS IN CELLS POORLY ATTACHED TO THE GROWTH SURFACE AFTER X IRRADIATION

Experiment No.	Dose (kr)	Time after irradiation (hrs.)	Number of cells examined	Per cent of nuclei that were:		
				Mitotic	Pycnotic	Resting
397	4.0	40–48	1537	45.4	35.3	19.4
		63–68	964	38.2	54.6	7.2
444	1.2	68–76	500	54.4	42.2	3.4
		106–112	46	22	63	15
	10.0	68–76	138	42	41	17
		106–112	74	46	36	18
436, 439, 442, 448, 450	0	—	2908	63.1 (57.5–66.4)	9.7 (2.9–18.6)	27.2 (21.5–36.2)

Growth medium was withdrawn at the times indicated, and the plate rinsed repeatedly with 5 ml. fresh medium or saline to dislodge the loosely attached cells. The harvested cells were sedimented on cover slips, fixed, stained with acetic orcein, and examined. Both mean values and ranges (in parentheses) are given for the control data.

they will account for the exponential nature of the terminal portion of the persistence curves. It is possible that the observed distribution of times of metabolic death reflects the natural life span for at least the more persistent of these cells, a concept that is inapplicable to a dividing population.

Growth of Individual Cells

Enlargement of HeLa S3-9 cells after exposure to 1.3 kr was previously shown to be exponential for several days, and to proceed at a rate more than one half as great as normal cell growth (Tolmach and Marcus, 1960). Other workers have reported that such extensive cell enlargement occurs only after X-ray doses falling within the narrow range of about 0.8 to 3.0 kr (Sheek *et al.*, 1960). Furthermore, using a different cell system, Kohn and Fogh (1959) reported an inverse relation between cell enlargement and dose, up to 3 kr.

Preliminary measurements of the dose dependence of HeLa S3-9 cell growth have yielded data in partial disagreement with these reports. As shown in

FIGURE 8, cell enlargement was slightly inhibited on increasing the dose 4-fold from 1.25 to 5.0 kr. It may be concluded that the manifold processes involved in the production of protoplasm from the growth medium are extremely radioresistant, in contrast to the division mechanism. The results of Whitmore *et al.* (1958) are consistent with this conclusion.

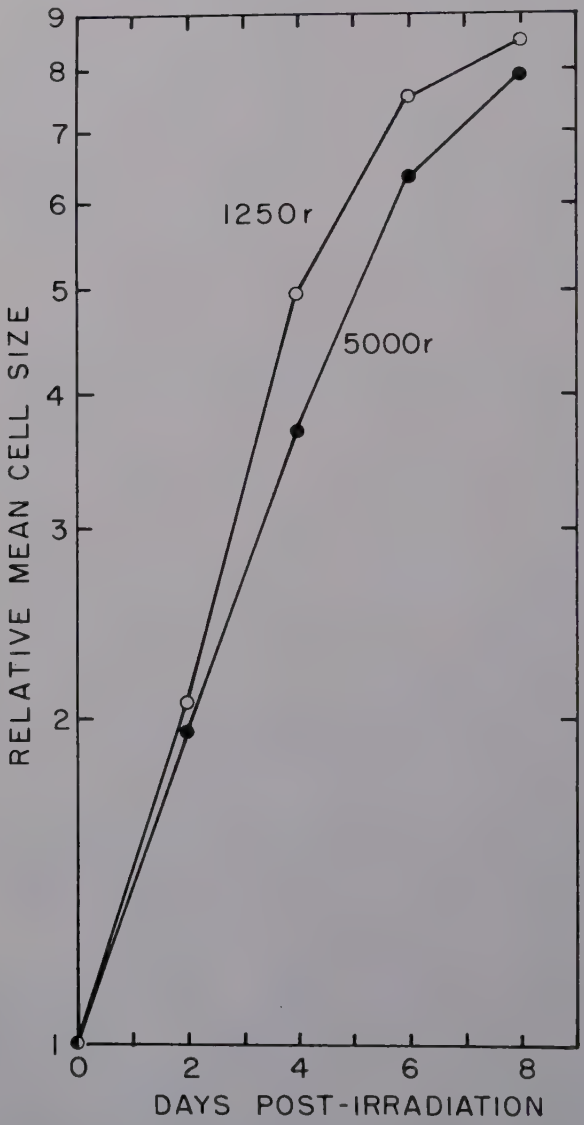


FIGURE 8. Growth of giant HeLa S3-9 cells following exposure to 220 kv. X rays. The mean size is plotted as a function of time after receiving doses of 1250 or 5000 r. Relative cell sizes were determined with the Coulter Cell Counter, the cells being suspended in phosphate-buffered saline with 0.2 per cent methylcellulose added to increase viscosity.

Conclusions

Measurement of the dose responses of the several parameters concerned with cell growth permits classifications of these cellular processes as either radiosensitive or radioresistant. In the former category are cell division, reproductive survival, and chromosome damage (Puck, 1960). The latter category includes general metabolic activity, cell enlargement and, possibly, the rate of cell division after radiation lag. Radiation-induced lag cannot be definitely classified but, since the effects of small doses can be detected, lag probably should be placed in the former category. Such classification is readily interpreted in terms of the concept that the primary radiation-induced lesion lies in the cell's genetic apparatus. The applicability of these findings to more normal cell types *in vitro*, and to *in vivo* cell systems remains to be tested.

Acknowledgments

I am grateful to Erich J. Diamant and Toyozo Terasima, who collaborated in the execution of certain of the experiments reported. Raymond A. Ritter, Jr., and Charlie Shaeffer carried out some of the experiments during tenure of Student Research Fellowships. Excellent technical assistance was provided by Mary Hasler and Allan J. Rossel.

References

Cieciura, S. J., P. I. Marcus & T. T. Puck. 1957. Virology. **3:** 426.
Engelberg, J. 1960. Abstracts of papers presented at the Fourth Annual Meeting of the Biophysical Society. : 37.
Ham, R. & T. Puck. Methods in Enzymology. In press.
Hood, S. L. & G. Norris. 1961. Radiation Research. **14:** 705.
Kohn, H. I. & J. E. Fogh. 1959. J. Natl. Cancer Inst. **23:** 293.
Lasnitzki, I. 1943. Brit. J. Radiol. **16:** 61.
Lea, D. E. 1956. Actions of Radiations on Living Cells, Second Ed. Chap. IX. Cambridge University Press. Cambridge, England.
Levine, S. 1960. Virology. **10:** 257.
Marcus, P. I. 1959. Virology. **9:** 546.
Marcus, P. I. & T. T. Puck. 1958. Virology. **6:** 405.
Puck, T. T. 1960. Progress in Biophys. **10:** 237.
Puck, T. T. & P. I. Marcus. 1956. J. Exptl. Med. **103:** 653.
Puck, T. T., P. I. Marcus & S. J. Cieciura. 1956. J. Exptl. Med. **103:** 273.
Sheek, M. R., R. M. DesArmier, B. P. Sagik & W. E. Magee. 1960. Exptl. Cell Research. **19:** 549.
Tolmach, L. J. & P. I. Marcus. 1960. Exptl. Cell Research. **20:** 350.
Tolmach, L. J., A. J. Rossel & R. A. Ritter, Jr. 1960. Radiation Research. **12:** 480.
Whitfield, J. F. & R. H. Rixon. 1959. Exptl. Cell Research. **18:** 126.
Whitmore, G. F., J. E. Till, R. B. L. Gwatkin, L. Siminovitch & A. F. Graham. 1958. Biochim. et Biophys. Acta. **30:** 583.
Whitmore, G. F., C. P. Stanners, J. E. Till & S. Guylas. 1961. Biochim. et Biophys. Acta. **47:** 66.

X-RAY EFFECTS ON CULTURED HUMAN AMNION CELLS*

Jørgen Fogh, June Lee Biedler, A. R. T. Denues

Division of Experimental Pathology and the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, N.Y. and the Sloan-Kettering Division, Cornell University Medical College, New York, N.Y.

The use of cell culture in experimental studies of X-ray effects has given valuable information that has led to a more thorough understanding of the effects on mammalian cells. Significant similarities, but also differences, between the response of mammalian cells and cells of lower organisms have been observed. Puck *et al.* have reported on the quantitative radiation effects on the reproductive capacity of single cells derived from a variety of human tissues and have found that the mammalian cell is the most sensitive "microorganism" described.[1,2] Epithelial and fibroblastic cells, of normal or malignant origin, in continuous culture or freshly isolated and polyploid and diploid cells have been studied.[1-4] For all these cells the close similarity of mean lethal dose (the dose necessary to reduce to 37 per cent the ability of the individual cells to multiply into a macroscopic colony[1]) has been impressive. The characterization of the giant cell developing in irradiated cultures has helped us to understand radiation effects also *in vivo*; it has given a new interpretation of the relationship between mitotic activity and X-ray sensitivity; and it has clarified various important problems involved in the definition of cell life.[5,1-4] Thus this report on our work with X rays, which started about three years ago in cooperation with H. I. Kohn,[6] should be considered chiefly as providing supporting observations.

Material and Methods

Using cells of the FL strain of human amnion,[7] experiments were designed to observe the effect of several X-ray doses on the growth and cell size, and to compare populations surviving X-ray exposure with those of the parent strain. The cells, derived originally from the normal human amniotic membrane of the placenta, have been characterized in many respects. Elaboration on their characteristics is unnecessary at this point. It will only be mentioned that, like cells of many other human strains, these cells now have a high chromosome number (hypotetraploid) and a high mitotic index and that they are tumor-producing in conditioned animals[8,9] and in human cancer patients.[10] The cells are highly susceptible to a variety of viruses,[11] and their structure and ultrastructure,[12] thoroughly studied, have been shown to be characteristically different from amnion cells in the native membrane[13] or in primary culture.[12]

Cells growing as monolayers in culture tubes were irradiated with either a Phillips RT-100 unit (with KO-100/8 tube) or a Picker, Vanguard unit (with two Machlett EG302 tubes). For the former the radiation factors were 100 kv, 8 mAmp., 23 cm. target-culture distance, beryllium-mica window; the total filtration by the glass wall was estimated as equivalent to 0.3 mm. Al. For

* The work reported in this paper was supported in part by Contract SA-43-ph-2445 from the Cancer Chemotherapy National Service Center, National Cancer Institute, Public Health Service, Bethesda, Md.

the latter the factors were 200 kv, 15 mAmp., 49 and 52 cm. target-culture distance with 0.4 mm. Cu filter. For each experiment the exposure dose rate within the culture tube was estimated by exposing a Victoreen ionization chamber within a glass tube under the same conditions used for irradiating the cultures.

Counts of the cell populations were made on single-cell suspensions prepared by versenation of the cultures at the day of and in the days following irradiation, up until day 10. With a hemacytometer the number of cells per tube and the diameter of a representative number of cells for each sample were determined. Cultures were fixed and stained in preparation for light and electron microscopy, usually 4 days after irradiation. The morphologic changes

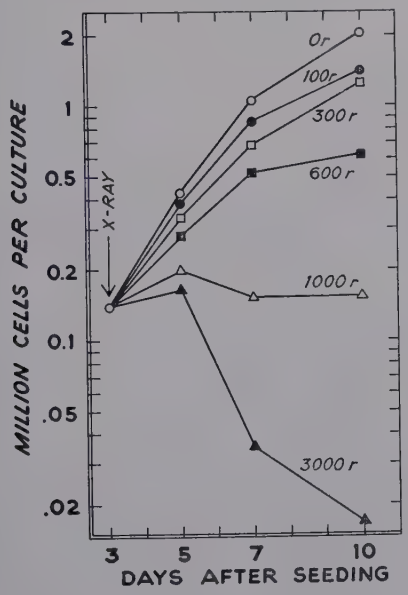


FIGURE 1. Cells per culture after irradiation on day 3.

described in this paper thus involve also many of the earlier changes seen in the cultures.

Results

The number of morphologically intact cells per culture at various times after irradiation on day 3 was found (FIGURE 1) to display a graded series of effects with the graded series of radiation doses. At the doses less than 1000 r the cell number increases up to day 10, while at 1000 r the number tends to remain constant; at 3000 r the number of cells decreases rapidly after the 2nd day.

The morphological changes are apparent from FIGURES 2*a* to *d*. Under phase microscopy, the unirradiated cells (*a*) and cells exposed to 600 r 4 days prior to fixation (*b*) appear very different. Most striking in the irradiated population is the increased cell size; both the cytoplasm and the nuclei appear enlarged, and the nucleolar material is prominent. In the normal cell, on an

average, 4 to 5 regularly shaped nucleoli are seen; in many irradiated cells the nucleolar material becomes concentrated into a few masses, or a single mass, usually of increased size.

Fields of monolayers having received 1000 and 3000 r (FIGURES 2*c* and *d*)

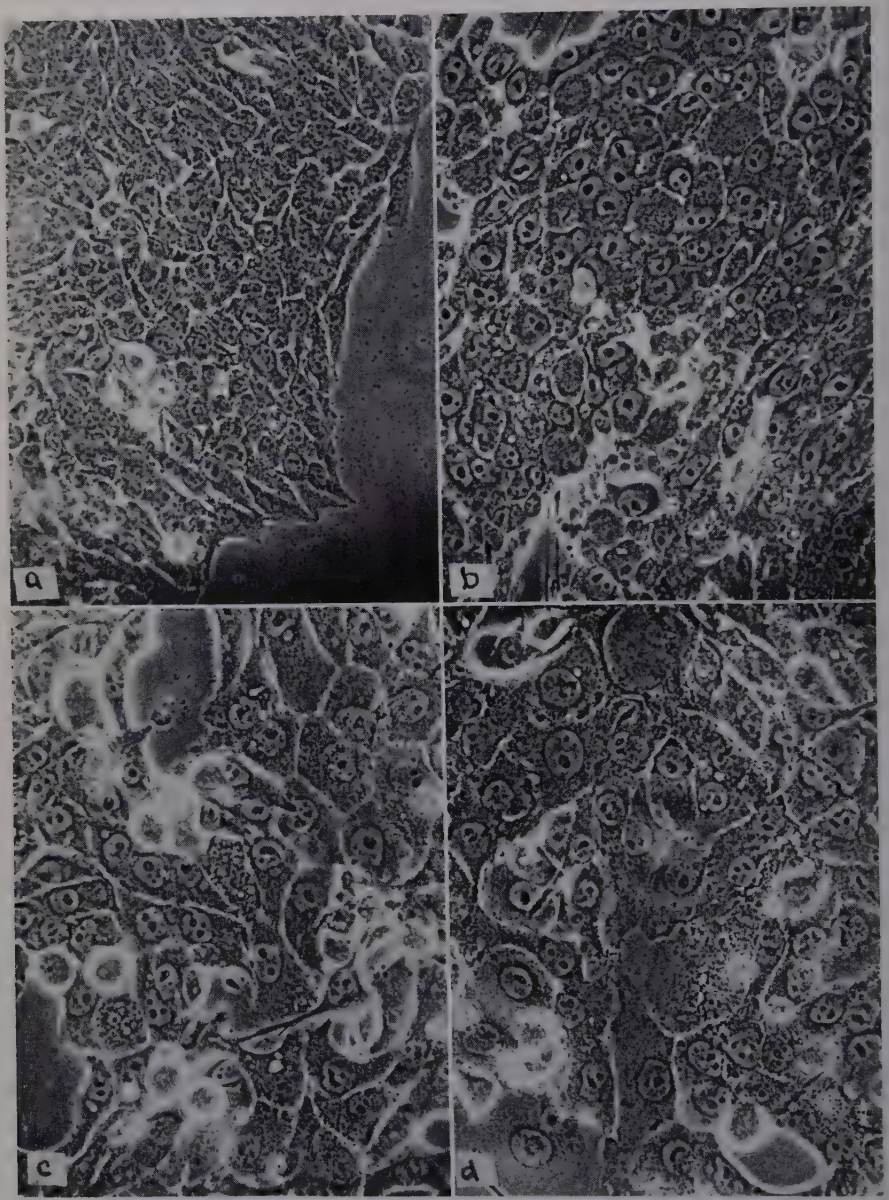


FIGURE 2. Fields of unstained FL cells under phase microscopy, ×178; (*a*) unirradiated; (*b*) four days after irradiation with 600 r; (*c*) 1000 r; and (*d*) 3000 r.

illustrate the much lower number of cells per field, the enlargement of the cells, and even more pronounced structural changes predominantly in the nuclei. The largest cells are giant cells. At this time after irradiation some of the cells have lost their ability to divide; others are growing slowly. Some of the cells, normal looking and still dividing, will die, perhaps after several divisions. In the culture having received 3000 r, all cells will die. A number of cells in the culture having received the highest dose have fallen off the glass. While mitotic figures are frequent in control cultures (5 per cent, or more), they become increasingly rare in the irradiated cultures with increasing X-ray dose. As pointed out, measurement of proliferation rate by mitotic index, however, is inaccurate in irradiated cultures.[5] Radiation causes abnormal mitosis;

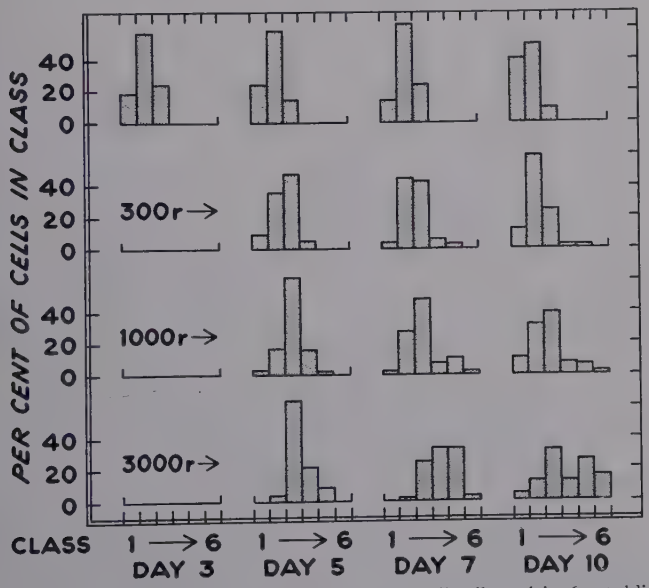


FIGURE 3. Cell volumes, after irradiation on day 3, distributed in 6 established classes; the smallest volume for each class was: class 1, 0.52; class 2, 2.25; class 3, 6.0; class 4, 12.5; class 5, 22.5; and class 6, 36.8. All $\times 10^{-6}$ mm^3.

many of the mitotic figures (20 to 30 per cent), however, are already abnormal in unirradiated FL cell cultures.

The distribution of cell volume was established from determinations of diameters of suspended cells (FIGURE 3). Six classes of volume were defined, the classes corresponding to minimum diameters of from 10.0 μ in class 1, and from 41.3 μ in class 6. Radiation was on day 3. Cells of the control cultures shown in the top row of the bar graphs belong within the 3 lowest distribution classes. On day 10, which corresponds to the 7th day after irradiation, there is a relative increase of cells of the smaller volumes in the control group, due to crowding. For all of the irradiated cultures at any time after irradiation, there is an increase in the size of cells. The shift in distribution was both toward larger volumes and toward a greater range of volumes, and was not an all-or-none phenomenon.

Increase in cell volume of irradiated cells was first noted by E. Paterson[14] in 1942 for chick fibroblasts. A varying degree of enlargement was seen even with the lowest doses of X ray employed. This observation is in general agreement with our results, as are results of studies of functional characteristics[1,2] and various nuclear anomalies observed in human giant cells.[3,4]

We have observed giant cells in the populations of irradiated FL amnion cells fixed 4 days after irradiation with doses of 600, 1000, and 3000 r. At this time the number has increased with the dose of X ray. Even after 3000 r the population does not consist exclusively of giant cells at this time but, as FIGURES 2*d* and 3 show, it contains cells with volumes within the range of volumes of the unirradiated population.

FIGURES 4*a* to *f* show, at higher magnification, unstained cells observed under phase microscopy for control culture (*a*), and for cultures exposed to 600 r (*b*), 1000 r (*c*), and 3000 r (*d*, *e*, and *f*). The nuclear anomalies in the irradiated cells are conspicuous; these include, characteristically, the apparently increased amount of nucleolar material in a larger nucleus, the irregular shapes of the nuclei, sometimes clover-leaf in shape, and cells showing extreme changes of the nuclear and nucleolar material that is broken up into multiple micronuclei of varying size, with or without any considerable increase in total cell size. In some cells it is a single large nucleus that is most characteristic.

Slides fixed with methyl alcohol and stained with van Gieson's stain show the changes very clearly. The enormous cytoplasm in the giant cell from a 600 r irradiated culture, the presence of 3 or 4 nuclei, the irregular shape of the individual nuclei with wrinkles of the nuclear membranes, and the strangely shaped nucleolar masses are all characteristic (FIGURE 5*b*). Small pieces of broken-off nuclear material are commonly observed, also noted by Pomerat *et. al.*[4] in many of the different cell strains they studied.

Giant cells with other morphological characteristics from 600-r irradiated cultures are shown in FIGURES 5*c* to *e*. Large vacuoles in the cytoplasm and nucleus have been observed in many of the irradiated FL amnion cells, such vacuoles previously found by Pomerat and his co-workers to be particularly characteristic of the Henle intestinal strain.[3] The numerous, mostly spherical nuclear masses of varying size are seen in FIGURES 5*d* and *e*. Note the nucleolar bud in *d*.

These giant cells, although they metabolize at a rate comparable to that of an equal mass of actively dividing cells, cannot multiply.[1] According to Puck's criteria,[2] the cell is already dead since, as a result of a lethal effect in the genetic apparatus, it is not able to multiply into a macroscopic colony. This effect may have been caused either in this particular cell or in one of its parents, up to several generations previously. Later stages of mitosis were not observed in giant cells. We can confirm this observation.

FIGURE 5*e* shows a giant cell with very numerous nuclear masses of ranging sizes. The picture gives the impression of an explosion of nuclear material[5] and causes one to speculate on the structural details of the micronuclei. It is indicated from this and the following figures that some are quite complete, but that all degrees of disintegration occur down to droplets of unrecognizable structure.

From cultures fixed with Dalton's chrome-osmium fixative[15] 4 days after

FIGURE 4. Fields of unstained FL cells under phase microscopy, ×612; (*a*) unirradiated; (*b*) four days after irradiation with 600 r; (*c*) 1000 r; and (*d*, *e*, and *f*) 3000 r.

irradiation with doses of either 600, 1000, or 3000 r, electron micrographs were made. As described, none of such populations consisted entirely of giant cells. In FIGURE 6*a* a budding of the nucleus is visible both on the nuclear surface and perhaps inside the sectioned nucleus. The double membrane of the bud

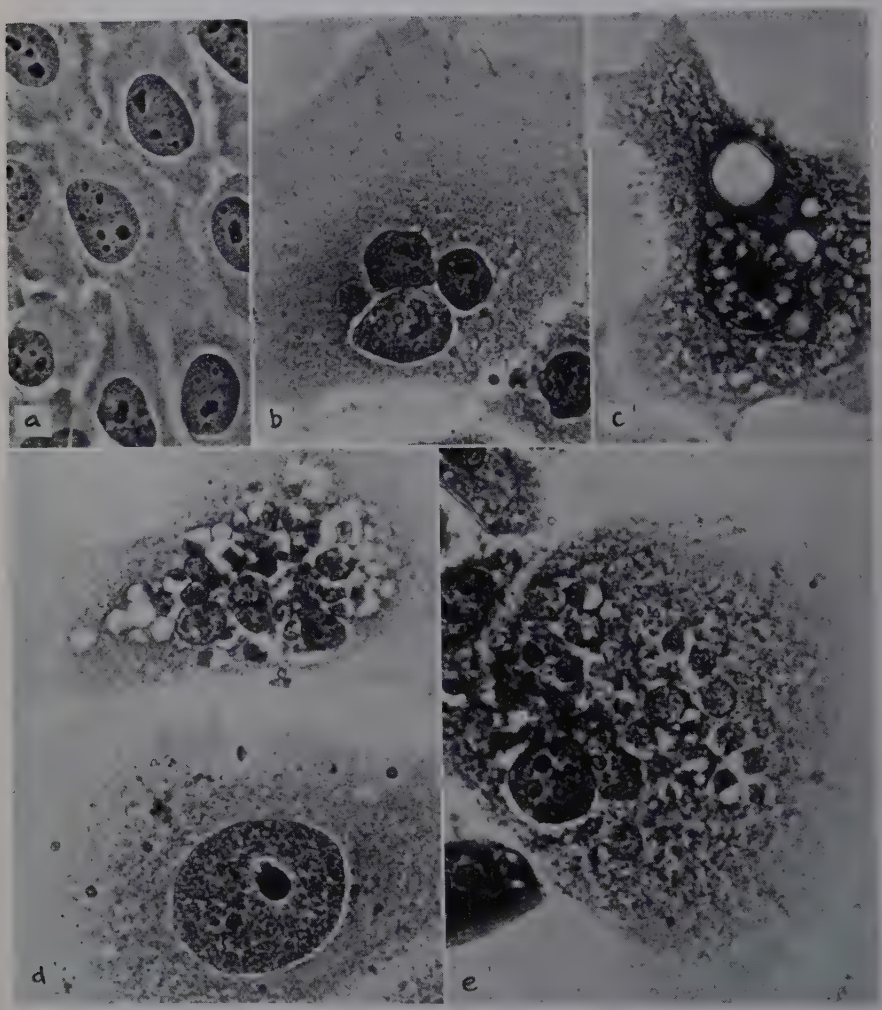


FIGURE 5. Van Gieson stained FL cells under phase microscopy; (*a*) unirradiated; (*b*, *c*, *d*, and *e*) giant cells from irradiated populations; (*a*, *b*, and *c*) ×566; (*d* and *e*) ×648.

is still contiguous with the main nuclear membrane. The bud membrane shows some irregular form that may lead to the formation of even smaller buds from this one. Notice in this picture the perinuclear areas that appear very light, empty, and essentially structureless. Such cytoplasmic areas, although occasionally observable in unirradiated cells, have been an outstanding feature in all the irradiated cultures.

The lobulation process is more pronounced in FIGURE 6*b*, the nucleus apparently being cut into a number of smaller nuclei. The low-density cytoplasmic area here surrounds closely most of the nucleus. Undoubtedly the damage in such areas can cause a complete separation of nucleus from cytoplasm, and almost nude nuclei may be seen.

A later stage of formation of multiple nuclei appears in FIGURE 6*c*. A variety of irregular nuclear forms of ranging size are mingled with cytoplasmic

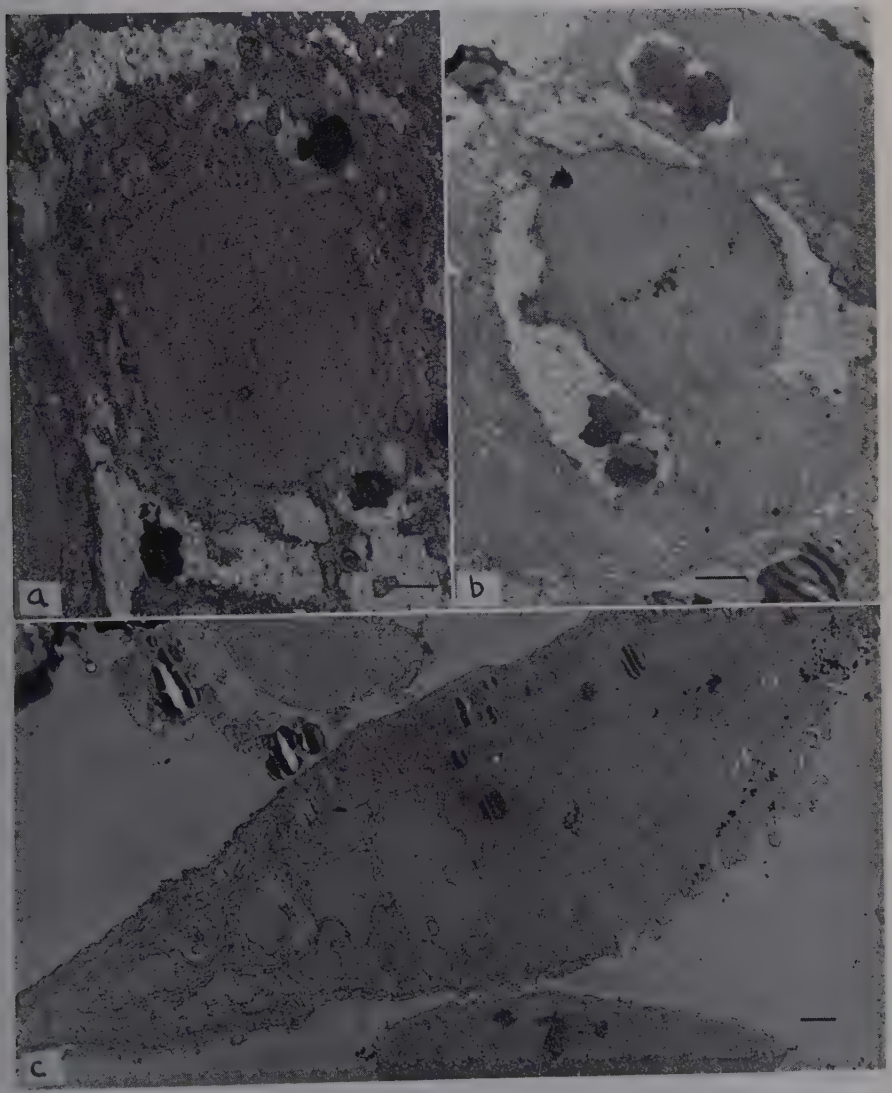


FIGURE 6. (*a*) Budding of the nucleus and perinuclear, structureless cytoplasmic areas in irradiated (1000 r) FL cell, ×6450; (*b*) more pronounced nuclear budding and lobulation, empty perinuclear area in irradiated (1000 r) cell, ×6450; and (*c*) irradiated FL cell (3000 r) showing multiple nuclei and nucleoli and prominent ergastoplasm, ×4540.

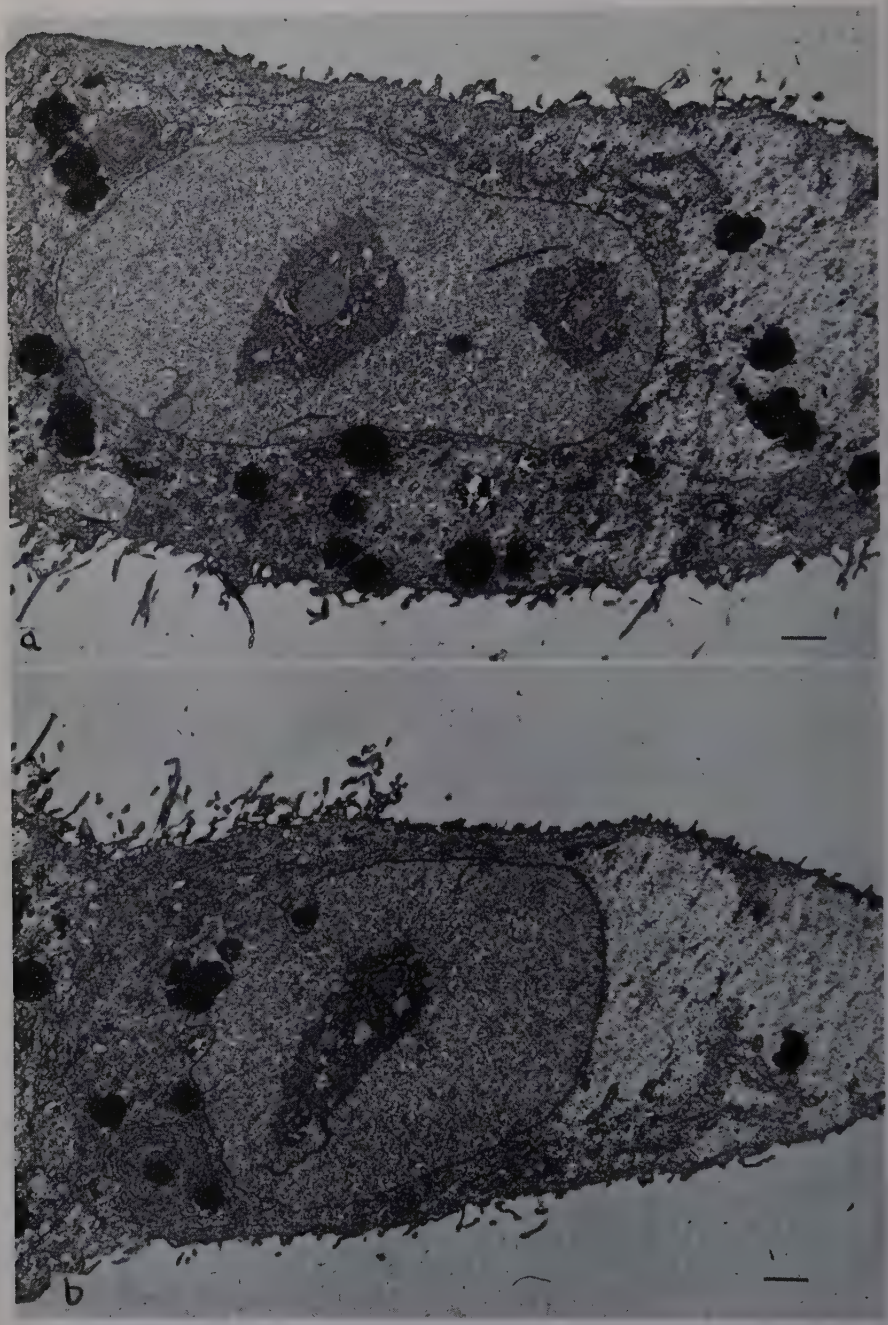


FIGURE 7. (*a*) Nucleolar changes in irradiated cells (3000 r). The large nucleoli contain a central, rather unstructured area, or (*b*) show unraveling. Note the light, structureless cytoplasmic areas, ×5460.

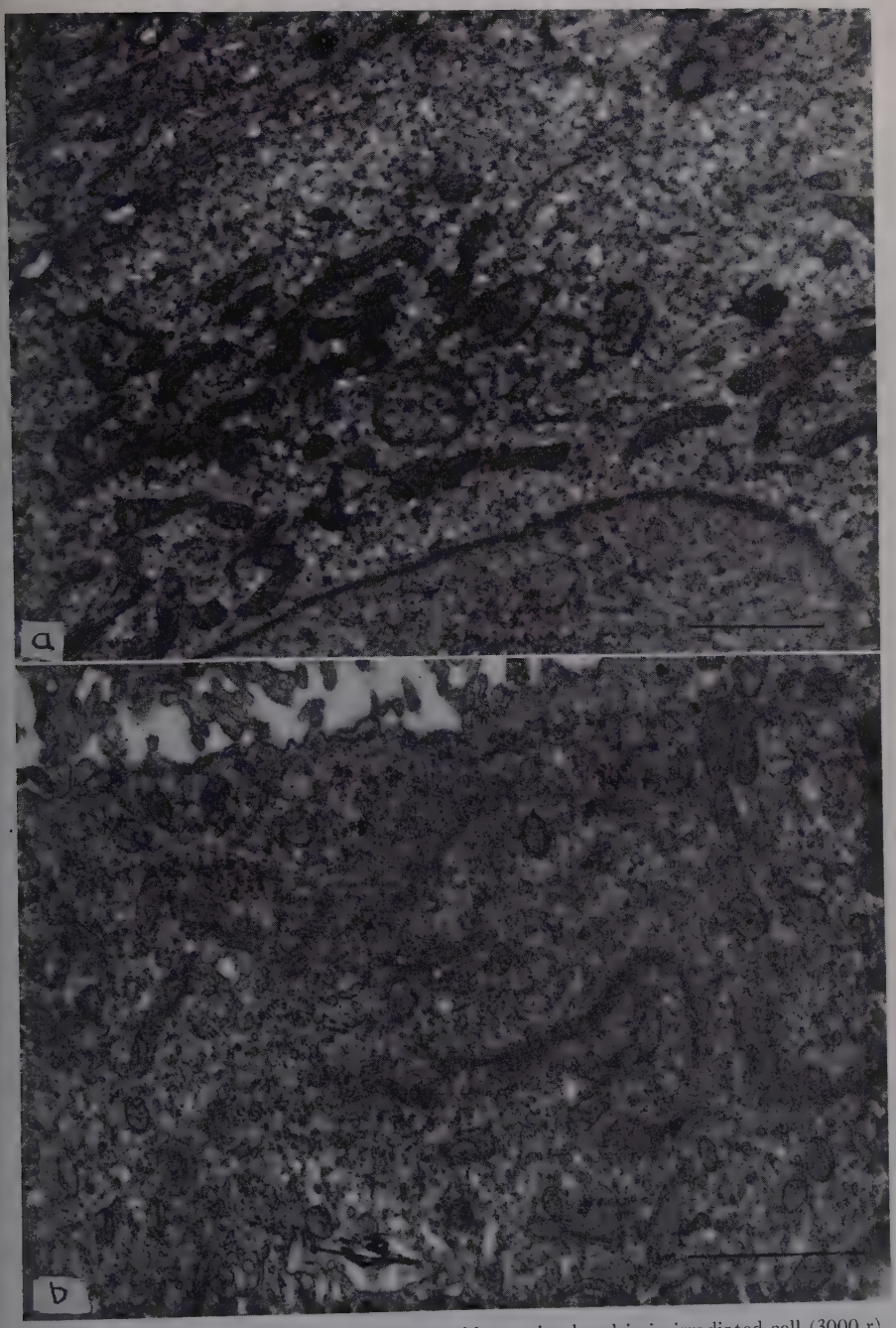


FIGURE 8. (*a*) Perinuclear concentration of long mitochondria in irradiated cell (3000 r), ×17,900; and (*b*) golgi regions in irradiated cell (600 r), ×23,300.

components. The prominent ergastoplasm here appears more developed than in the normal cell. Small areas of nucleolar material are present.

An alteration of the nucleolar material frequently observed in the irradiated cells is shown in FIGURE 7*a*. The nucleolar mass, increased in size, includes,

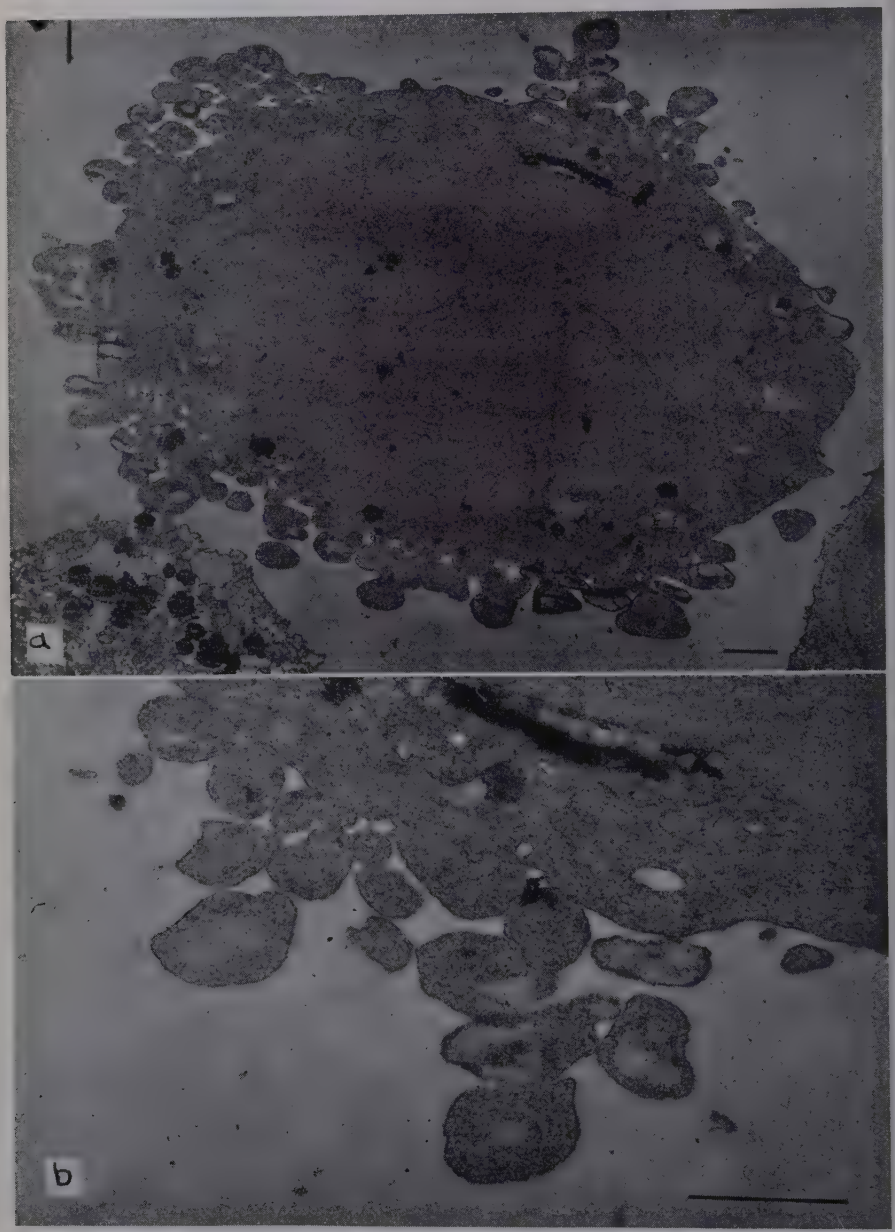


FIGURE 9. (*a*) ×6600. The surface of an irradiated cell (600 r) gives off membrane-surrounded bubbles that may contain well-preserved structures. (*b*) ×20,700.

in addition to the threadlike structures, a central area of relatively unstructured light material. The nucleolar material may take all sizes; in the enlarged nuclei it may be large (FIGURES 2*d*, and 4*b*, *c*, *d*, and *f*), while in the multinucleate cells it may or may not be seen within each of the micronuclei (FIGURES 5*d* and *e*).

In one cell, unusual evidence suggesting unraveling of the nucleolus was apparent (FIGURE 7*b*).

Another distinguishing feature observed by electron microscopy of the irradiated cells is the perinuclear concentration of long filamentous mitochondria,[4] often placed in the rather structureless cytoplasmic matrix (FIGURE 8*a*). This figure demonstrates how certain of the cytoplasmic components, notably the mitochondria, seem very well preserved despite the irradiation, although the cytoplasmic matrix has changed considerably toward the homogeneous

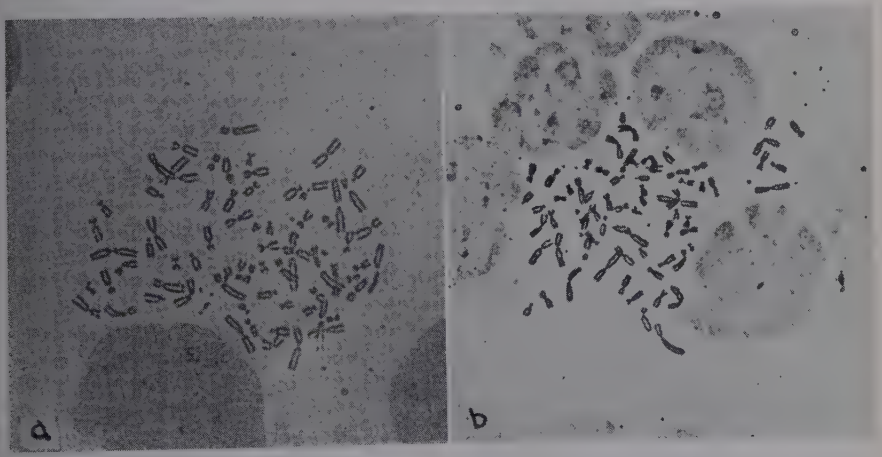


FIGURE 10. Chromosomal constitution of unirradiated FL cells; acetic orcein stain (*a*), ×718; and (*b*), ×560.

structureless state. Well-preserved Golgi regions have also been observed in the treated cells (FIGURE 8*b*).

A sudden dissolution of the cytoplasm from an irradiated multinucleate cell is shown in FIGURE 9*a*. Here many bubbles surrounded by membranes are apparently being given off from the cell surface; these may contain well preserved structures, particularly whorls of double membranes (FIGURE 9*b*).

Increased resistance to X ray of L strain mouse cells repeatedly exposed to high doses of X rays has recently been reported.[16] The resistant cell lines had a lower average chromosome number, and a large metacentric chromosome was almost always lacking. From our experiments with human cells,[6] it was evident that there was no striking difference between the X-ray sensitivity or growth behavior of unirradiated cultures and of cultures exposed once or twice to 1000 r, from which the surviving cells were recovered and grown up to a new stock. The only noticeable difference was in the length of the lag period for cell division. Similar results were obtained by Puck;[2] although mutants with morphologic, nutritional, and chromosomal differences were easily estab-

lished from irradiated cultures, the X-ray sensitivity of mutants approximated closely that of the parent strain. Neither did Bases[17] find permanent change in growth rate or response curve of survivors from a human cell line subjected to as many as 8 serial irradiations of 500 r each. The observed increased resistance for the mouse cells, if confirmed, is important, and indicates the necessity of continued studies of this problem.

Chromosomal alteration was here found in X-irradiated FL cells of mono-

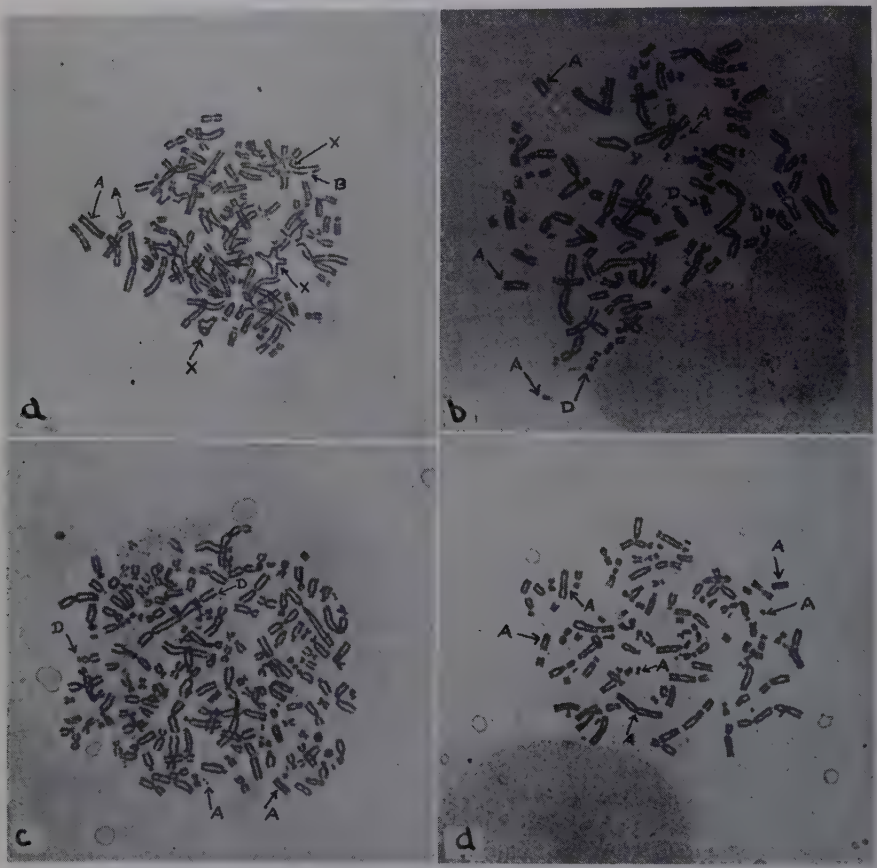
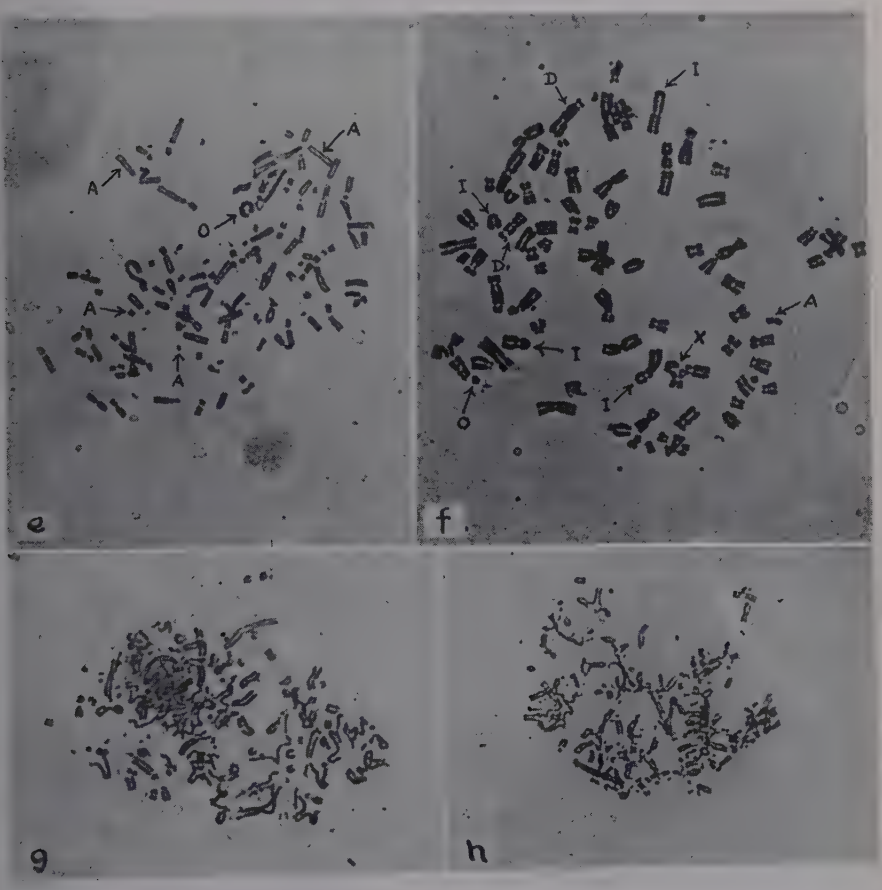


FIGURE 11. (*a*, *b*, and *c*) Damaged chromosomes of irradiated FL cells, 600 r; (*d*, *e*, and chromatid breaks (*B*), and translocational exchanges (*X*). (*b*): large and small acentric fragments (*A*); and dicentric chromosomes (*D*). (*d*): marked number of acentric fragments with diplochromosomes showing acentric fragments (*A*); dicentric chromosomes (*D*); intratreme chromosomal damage.

layers treated with colchicine and stained with acetic orcein 4 days after exposure to 600 r, 1000 r, or 3000 r. The chromosomal constitution of the unirradiated cells (typified in FIGURES 10*a* and *b*) includes many departures from the normal human karyotype, not only in number (which varied between 69 and 75) but also in chromosomal varieties. In general the radiation-induced changes

were of the same types in the differently irradiated cultures; the frequency of changes, however, was obviously increased with dose of radiation. The changes, ranging from simple chromosome breaks to marked degree of fragmentation and complicated structural rearrangements, are exemplified in FIGURES 11*a* to *c* (600 r), FIGURES 11*d* to *f* (1000 r), and FIGURES 11*g* and *h* (3000 r). Acentric fragments (*A*) of varying size (FIGURES 11*a* to *f*, particularly marked in FIGURES 11*d*, and *e*), chromatid breaks (*B*, FIGURE 11*a*), and dicentric



f) 1000 r; and (*g* and *h*) 3000 r. Acetic orcein stain, all ×716. (*a*): acentric fragments (*A*); fragments (*A*); and dicentric chromosomes (*D*). (*c*): a cell of higher ploidy showing acentric (*A*). (*e*): marked number of acentric fragments (*A*); and ring chromosome (*O*). (*f*): a cell changes (*I*); ring chromosome (single; *O*), and translocational exchange (*X*). (*g* and *h*) ex-

chromosomes (*D*, FIGURES 11*b*, *c* and *f*) were frequently observed. Other configurations showed translocational exchanges (*X*, FIGURES 11*a* and *f*) and intrachanges (I, FIGURE 11*f*). Ring chromosomes (*O*) were likewise seen as an effect of irradiation (FIGURES 11*e* and *f*). Cells of higher ploidy (FIGURE 11*c*) and even the formation of complete diplochromosomes (doubling of the number

found normally in the unirradiated cells) were observed in the irradiated populations (FIGURE 11*f*). Extreme chromosome damage with bizarre alterations was frequently seen in the cultures that had received the highest doses of irradiation (FIGURES 11*g* and *h*).

Chromosomal aberrations have been found in cultured diploid human cells irradiated with as little as 25 or 50 r,[18] and it is indicated[1,2] that chromosomal damage is the primary site of animal-cell radiation injury.

Conclusion

The early effects of 100 kv X rays on monolayers of continuously grown human amnion cells of strain FL include loss of continuity of the cell sheet, reduction in number of cells, gradual increase in cell size, and decrease in occurrence of mitotic figures. For doses examined in the range 300 to 3000 r, the increase in cell size was accompanied by a greater range of volumes. Cell enlargement, interpreted as continued cellular growth under the condition of inhibited cell division, was reflected in the nucleus as well as in the cytoplasm. The largest giant cells were present 4 days after irradiation. Mitotic figures did not occur in giant cells. Cytoplasmic changes in the irradiated cells included enlargement, vacuolization, and formation of empty-looking, rather structureless areas, mainly perinuclear, that may figure in the separation of cytoplasm and nuclei. Prominent ergastoplasm and well-preserved mitochondria and Golgi complexes were seen within the irradiated cells, but a bubbling at the cell surface with suggested blebs containing well-preserved endoplasmic reticulum suggested a sudden dissolution of the cell. Nuclear anomalies included increased number of nuclei as well as marked changes in size and shape. Budding and break-off of nuclear material, wrinkling, and irregularities of nuclear membrane, lobulation, and vacuolization were frequently observed. The multinucleate cells occurred frequently and, apparently as a late stage, the nuclei seemed to explode into numerous spheroidal masses. The nuclear RNA was seen to take all sizes and, especially at the earlier stages of change, was characteristically in the form of irregular enlarged nucleoli. A central zone of relatively structureless material was often observed within the nucleoli and some evidence of their unraveling was seen. In multinucleate cells, small nucleoli were seen within some micronuclei. Surviving cells from cultures exposed once or twice to 1000 r showed no striking difference in growth behavior or X-ray resistance from the parent culture. The chromosomes of FL cells displayed responses to radiation that ranged from simple breakage to marked fragmentation, with increased dose of radiation, and included complicated structural rearrangements.

Acknowledgment

We gratefully acknowledge the assistance of Frances C. Mottram and Elliott H. Stonehill.

References

1. PUCK, T. T. & P. I. MARCUS. 1956. Action of X rays on mammalian cells. J. Exptl. Med. **103:** 653–666.
2. PUCK, T. T., D. MORKOVIN, P. I. MARCUS & S. J. CIECIURA. 1957. Action of X rays

on mammalian cells. II. Survival curves of cells from normal human tissues. J. Exptl. Med. **106:** 485–500.
3. Pomerat, C. M., S. P. Kent & L. C. Logie. 1957. Irradiation of cells in tissue culture. I. Giant cell induction in strain cultures versus elements from primary transplants. Zeitschr. f. Zellforschung. **47:** 158–174.
4. Pomerat, C. M. 1958. Cellular changes induced by radiation. Ann. N.Y. Acad. Sci. **71:** 1143–1155.
5. Stroud, A. N. & A. M. Brues. 1954. Radiation effects in tissue culture. Texas Repts. Biol. & Med. **12:** 931–944.
6. Kohn, H. I. & J. Fogh. 1959. Some prompt and delayed effects of X rays on the growth of human amnion cells in tissue culture. J. Natl. Cancer Inst. **23:** 293–304.
7. Fogh, J. & R. O. Lund. 1957. Continuous cultivation of epithelial cell strain (FL) from human amniotic membrane. Proc. Soc. Exptl. Biol. Med. **94:** 532–537.
8. Fogh, J. & K. Hok. 1958. Tumor production in X-ray and cortisone-treated rats given injections of human cells in continuous cultivation. Cancer Research. **18:** 692–697.
9. Friedman, M. & J. Fogh. 1958. Evaluation of malignancy of tumors produced in X-irradiated and cortisone-treated rats injected with human cells in continuous culture. Cancer Research. **18:** 879–886.
10. Moore, A. E. 1958. Tumorigenic activity of cultures. Ann. N.Y. Acad. Sci. **76:** 497–5.05.
11. Fogh, J, R. O. Lund & M. McClain. 1960. Sensitivity of FL cells to polio and adenoviruses. Arch. ges. Virusforsch. **9:** 559–570.
12. Fogh, J. & G. A. Edwards. 1959. Ultrastructure of primary amnion cells and transformed FL cells in continuous culture. J. Natl. Cancer Inst. **23:** 893–923.
13. Edwards, G. A. & J. F.ogh. 1959. Micromorphologic changes in human amnion cells during trypsinization Cancer Research. **19:** 608–611.
14. Paterson, E. 1942. A comparison of the action of X and gamma radiation on fibroblasts. Brit. J. of Radiology. **15:** 257–263.
15. Dalton, A. J. 1955. A chrome-osmium fixative for electron microscopy. Anat. Record. **121:** 281.
16. Rhynas, P. O. W. & H. B. Newcombe. 1960. A heritable change in radiation resistance of strain L mouse cells. Exptl. Cell Research. **21:** 326–331.
17. Bases, R. E. 1959. Some applications of tissue culture methods to radiation research. Cancer Research. **19:** 311–315.
18. Bender, M. A. 1957. X-ray induced chromosome aberrations in normal diploid human tissue cultures. Science. **126:** 974–975.

ATTEMPTS AT INFLUENCING THE RADIATION RESPONSE OF HUMAN TUMOR STRAINS A.Fi. AND HeLa IN CONTINUOUS CULTURES: A TEST OF SYNKAVIT (SYNTHETIC VITAMIN K) AS "RADIOSENSITIZER"*

Annelies Schleich,† Margaret K. Gey, George O. Gey

Finney-Howell Cancer Research Laboratory, Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, Md.

Interest in the possible enhancement of radiation effects led us to explore several procedures on tissue-culture strains of human tumor cells. The reports[1-7] of Mitchell and Simon-Reuss on the modification of the effect of X rays by the preliminary administration of Synkavit (synthetic vitamin K) to tissue cultures of chick fibroblasts, to rats bearing Walker rat carcinoma 256, and to patients, especially those afflicted with bronchogenic carcinoma, constitute a formidable effort in the search for more effective ways of improving radiation therapy of tumors. In their *in vitro* studies, considerable emphasis has been placed upon an extensive analysis of the mitotic effects of the radiations in conjunction with the administration of Synkavit. These interpretations were for the most part limited to the responses in the first 24 hours. Concentrations used vary between 2, 3, and 4×10^{-6} *M* followed by X rays between 150 r and 300 r.

Mitchell and Simon-Reuss have also reported the persistence of radiosensitivity for 5 weeks on 16 subcultures of chick fibroblasts treated originally with Synkavit for 24 hours. This would indicate a most remarkable preservation of a "sensitive" state through many cell generations.

We have explored some aspects of the responses of cells to radiations and are now reporting the effects of Synkavit when used alone and in combination with X rays. The availability of two preparations of Synkavit‡ made it necessary to evaluate responses of continuous cultures at various doses for different periods. These observations were carried out on continuous cultures of the fibrosarcoma strain A.Fi. and the HeLa strain of epidermoid carcinoma. In the present experiments we have studied the mitotic changes and the general responses of these two human malignant strains under the influence of Synkavit after short intervals of treatment and for varying periods up to several months. The protracted treatment with Synkavit was carried out to test the tolerance of these two strains. Our results gave evidence of a difference in cell response to the two types of Synkavit. Possible enhancement of radiation effects in tissue culture was carefully evaluated.

* The work described in this paper was supported in part by a grant-in-aid from the American Cancer Society, New York, N.Y.; by Research Grant C-356 from the National Cancer Institute, Public Health Service, Bethesda, Md.; and by a grant from Hoffmann-La Roche, Inc., Nutley, N.J.

† Fellow in Surgery, 1954–1955; visiting Fellow 1956, Finney-Howell Cancer Research Laboratory. Present address: Institut für experimentelle Krebsforschung, University of Heidelberg, West Germany.

‡ The Synkavit was kindly made available by S. Evert Svenson of Hoffman-La Roche Inc., Nutley, N.J. The "A" Synkavit "Roche" Lot 107, in powder form, was made at the Hoffman-La Roche Inc., Nutley plant, and "B" Synkavit R23 aqueous solution was made at its Welwyn, England plant.

Material and Technique

Cell strains. The human fibrosarcoma strain A.Fi. was first explanted *in vitro* on April 25, 1938, in G. O. Gey's laboratory at Johns Hopkins Hospital and Medical School, Baltimore, Md.

The patient was a 66-year-old white male. His tumor had partially destroyed the head of the humerus. Microscopic study of H. and E. sections revealed an exceedingly cellular fibrosarcoma (FIGURE 1*a*).

The cultures were prepared from roller-tube cultured stocks in fluid media with cells grown directly on glass. This fluid medium consisted of 50 per cent human placental cord serum and 50 per cent balanced salt solution (Gey).

Culture preparations were set up on 7 × 25 mm. No. 1 tube slips and incubated in roller tubes with 12 drops of *5050* medium. In most cases four 1-mm. fragments of the cell colonies were placed on the tube slip and an additional 4 fragments on the adjacent tube wall for comparative study.

Following various periods of growth and with change of the medium twice weekly, the tube slips were removed from the roller tubes, rinsed momentarily at 37° C. in balanced salt solution, fixed in Bouin's solution, and stained with Mayer's hemalum.

Cytology of A.Fi. Fibrosarcoma Cells in Vitro

The original tissue used for culture almost from the beginning produced fairly thin, more or less rounded tissue-culture colonies.[17] Outgrowth for the most part consisted of a single layer of cells (FIGURE 1*b*) spread out on the glass. Although the sections revealed elongated cells, in the cultures these sarcoma cells varied morphologically from irregular spindle types to somewhat polygonal types. The cytoplasm was usually expanded and sometimes extremely thin.

The great variation in nuclear morphology of the A.Fi. strain is worthy of description. The persistent kidney or C shapes are apparently also derived from the original daughter-cell configuration of the chromosomes (FIGURE 2*b*). Direct observations show the nuclei to vary in size, often by factor 10 or more. Some of the nuclei are quite rounded, others may be greatly elongated and, in some areas, exceedingly bent, even to the formation of ring forms. Other shapes include greatly lobed nuclei, with extreme forms suggesting a pinching-off process that may perhaps help to account for the production of some multinucleated cells. Stained preparations show in many instances fine bridges between the almost completely pinched-off nuclei. Frequently some of these pinched or constricted nuclear units show no demonstrable nucleoli. The pinched-off forms are more frequent than strictly multinucleated cells. There are, however, cells present with several large polyploid nuclei. Other cells show few to many micronuclei in addition to the larger nuclei.

Partial constriction of the nuclei or indentation of the membrane may be seen in both living and fixed cells. Continuous observations on individual living cells show considerable change in nuclear form from the highly indented to more or less rounded states. Nuclear rotation also occurs. These phenomena are shared by many cultured cells.

The juxtanuclear area that can become greatly enlarged under stress usually obscures a portion of the nucleus, and consists in the living cells of more or less

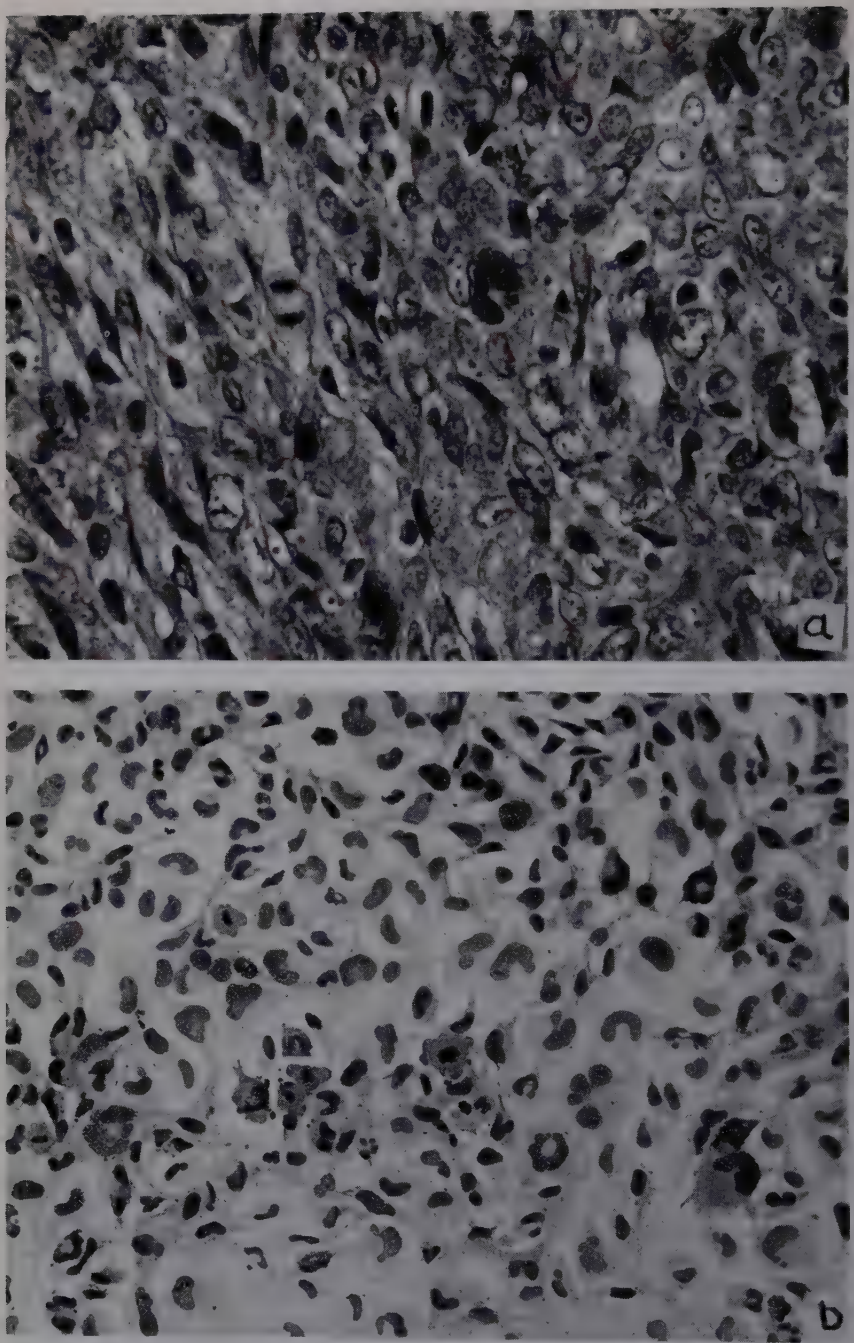


FIGURE 1. Human fibrosarcoma strain A.Fi. (*a*) H. and E. stained section of original tumor; and (*b*) low-power image of stained tube slip. Control culture. Note pleomorphism of nuclei, especially indented and bent forms. Mayer's hemalum.

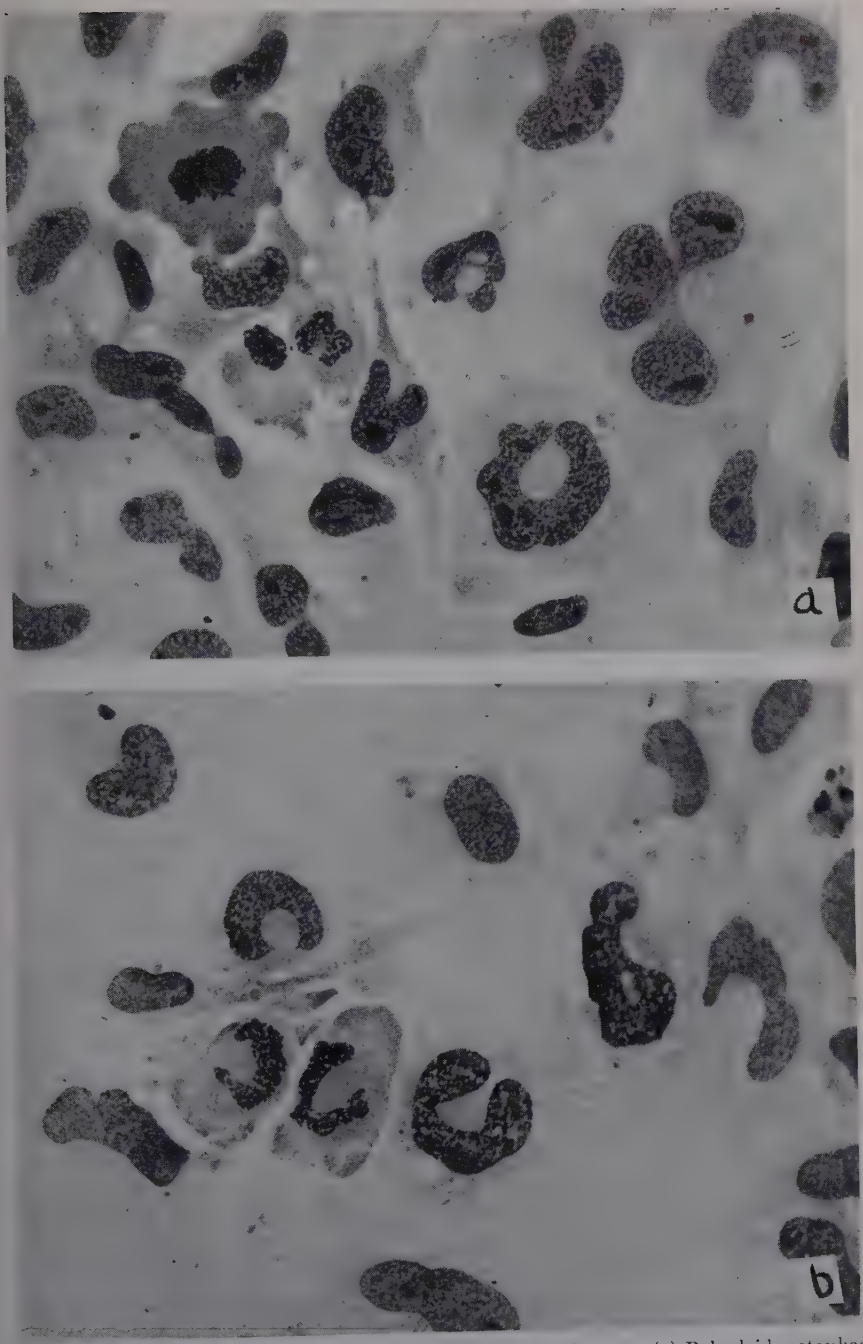


FIGURE 2. Human fibrosarcoma strain A.Fi. control cultures. (*a*) Polyploid metaphase and average diploid telophase; various forms of "pinched-off" nuclei; and (*b*) hyperdiploid telophase with horseshoe-shaped arrangement of the chromosomes. Mayer's hemalum. ×500.

rounded droplets of different sizes and appearance with some mitochondria interspersed. A distinct centrosome has not been seen. Occasionally the mitochondria may be seen radiating from the juxtanuclear area. Living-cell observations with phase microscopy may show nuclei with an indentation in this area. This suggests a definite relationship of the juxtanuclear mass to the indentation. This mass may be concerned with active transport of material between nucleus and cytoplasm. The indentation may be due to the more rigid character of the juxtanuclear mass on which the softer nucleus lies. The elastic character of the A.Fi. nuclei is revealed by the great changes in nuclear shape apparently induced also by cell movement, as seen in motion picture analyses.

The nucleoli of the interphase cells are generally irregular in number, size, and staining properties. Many of them are rounded and prominent. These may however expand considerably to produce elongated or scattered forms.

The mitotic display of this strain has its constant characteristics. The cultures have a fairly rapid growth rate, and mitoses are frequent. The multipolar forms and those with an irregular chromosomal distribution do not exceed 3 per cent of the total mitotic count. Among the multipolar forms the tripolar divisions are most frequent.

The shape of the giant hyperchromatic nuclei and also the nucleoli may be seen to persist clearly during prophase. These usually produce a giant metaphase plate following a long period of organization and, finally, accomplish a bipolar cleavage with high polyploid chromosomal sets. After cleavage the telophase-chromosome groups often retain a distinct horseshoe shape and eventually contribute to the bent shape of the interphase nucleus.

The HeLa cultures,[2-16] maintained in similar media, were derived from one of our original HeLa strains. These were isolated February 1951, from a small, invasive lesion of the cervix uteri of a 30-year-old colored patient. The diagnosis was "early invasive transitional cell carcinoma." Within 6 mo. following biopsy the patient died of extensive metastasis. This strain grows usually as a monocellular epithelial sheet on glass in fluid cultures. It can be grown in dispersed form after trypsinization and in agitated fluid with cells in suspensions.[18-23] A variety of sublines and some single cell clones exist.

Since the responses to radiation affect profoundly the mitotic behavior, evaluations of the mitotic changes were of special interest since the HeLa strain displays a very wide variety of irregular polyploid mitoses resulting in great differences in cell and nuclear size and the number of nuclei. Some characteristics of mitosis of various HeLa cells in culture have been reported.[24-27] Observations were made in the same manner as those reported in the experiments with A.Fi.

Technique of Radiation

The roller-tube cultures (16 × 150 mm. Pyrex brand) with or without tube slips were irradiated with X rays on a rotating device at 12 rpm in such a way as to permit a more or less uniform exposure. The colonies were allowed to remain immersed in the culture medium during irradiation and with continued incubation at 37° C. The irradiated fluid was removed the following day. The dose rate varied from 80 r/min. to 169 r/min., depending on the dose re-

quired, and was delivered by a Kelikett X-ray machine run at 250 kv at constant potential (V) with an added filter of 1 mm. Al and 1.98 mm. HVL Cu. The aperture was controlled with a safe margin beyond the tubes by observing them with a TV observation unit during the irradiation. Exact readings were taken before each irradiation with the National Bureau of Standards calibrated Victoreen 4-meter, 250 r black nylon ionization chamber. The readings were taken in international roentgens corrected to 0° C. and 760 mg. Hg.

Effects of Synkavit on HeLa Cells

Synkavit solutions are unstable under the conditions of incubation. Refrigerated stock was therefore used in various experiments, and fresh solutions were made up for each experiment.

The experiments carried out on the effects of "A" (American) Synkavit and "B" (British) Synkavit involved the use of HeLa cultures with 4 colonies grown directly on the tube wall and 4 on tube slips (10 × 32 mm.). These were set up in flat window tubes (Gey) and maintained in 15 drops of medium *5050*. The effects were evaluated at 12, 18, 24, 36, and 48 hours, at which time tube slips were removed for fixation. In order to provide information on the effects of protracted treatment with Synkavit, the remaining colonies left on the tubes and suitable controls were continuously cultured with regular fluid renewals and with fresh Synkavit treatment twice weekly.

Some Results

Some preliminary experiments were carried out on HeLa cells grown directly on glass in fluid medium *5050*. The concentrations varied between 4×10^{-4} M and 4×10^{-8} M. These experiments revealed a marked toxicity at the highest concentrations and no discernible effect at the lowest concentration during 48 hours of observation. Additional experiments were therefore limited to concentrations between 4×10^{-5} M and 4×10^{-7} M. All concentrations showed at 6 and 12 hours a slight granularity and contraction of the living cells and colonies. However, at 18 hours more striking effects were found. At this time, especially at the highest concentration (4×10^{-5} M) for both preparations, considerable mitotic arrest was present, with chiefly pyknotic metaphase cells exceeding in number the normal metaphases found in the controls. No postmetaphase stages were present but were found at 6 and 12 hours and, at this time, they were in fairly good condition. At 24 hours an apparent recovery had taken place. New anaphases and telophases could be found, but the mitotic index was reduced as compared to the controls. The effects for the same time intervals at concentrations of 4×10^{-6} M and 4×10^{-7} M were proportionately decreased.

A comparison of the A and B Synkavit demonstrated that the A Synkavit appeared to produce cellular lesions earlier and, apparently, more severe. The chromosomes of the mitotic figures were more swollen, sticky, and clumped than those treated with B Synkavit. These responses changed strikingly following protracted treatment. After 10 days the cultures continued to survive in a more or less contracted state. Injury was especially marked at 4×10^{-5} M of B Synkavit. Here there was still a mitotic arrest with many large rounded cells in metaphase. The intermitotic cells were granular and swollen,

but they seemed to be active. At 4×10^{-6} *M* the damage was less, but metaphase arrest was still prominent. Some postmetaphase stages however indicated progressive mitosis. The cultures that had received B Synkavit at 4×10^{-7} *M* appeared active and healthy although still somewhat contracted.

In striking contrast to the cultures treated with B Synkavit, those treated with A Synkavit appeared to show much less injury. At 4×10^{-5} *M*, for instance, there was only some clumping of the metaphase chromosomes without any remarkable degeneration. The cultures treated with 4×10^{-6} and 4×10^{-7} barely differed from their controls.

These differences between the effect of A and B Synkavit persisted during protracted treatment. This may be explained on the basis of the source material that was used. One of them (B Synkavit) was available as a solution that may have contained a preservative. The other (A Synkavit) was available as a powder from which aqueous solutions were prepared.

During the time of protracted cultivation, a certain degree of tolerance toward Synkavit seemed to develop in the HeLa cells. However, the retardation in growth, especially at the concentration of 4×10^{-5} *M* and 4×10^{-6} *M*, was obvious, although all of them could have been grown indefinitely.

Effects of Synkavit and X rays on the HeLa strain. For our experiments with Synkavit and X rays, we used cultures that had been under protracted treatment with Synkavit A or B for 51 days at concentrations of 4×10^{-5}, 4×10^{-6}, and 4×10^{-7} *M*. In order to approximate some therapeutic dosages, we gave 2700 r X rays to each group receiving the various concentrations of Synkavit and to 1 control group without the Synkavit pretreatment. The cultures were fixed at 30 hours and at 7 days following irradiation. Since the differences in response between the 2 types of Synkavit in combination with X rays were not remarkable, we are reporting the general results of this combined experiment.

The Synkavit-treated colonies showed at concentrations of 4×10^{-5} and 4×10^{-6} a smaller area of outgrowth than the controls. A concentration of 4×10^{-7} produced very thinly spread-out colonies. Counts showed that all concentrations had about the same mitotic index and about the same distribution of the various phases of mitosis as found in the controls. The retardation of outgrowth would appear to be due to a prolonged mitotic course. The abnormal mitotic pictures (polyploidy, irregular movement, stickiness of the chromosomes, and giant cell formation) did not exceed the spectrum found in the HeLa controls at any time. The interphase cells had, after the protracted treatment with Synkavit, still more vacuoles in the cytoplasm and even in the nuclei when compared with the controls.

The colonies irradiated with 2700 r X rays were severely damaged. The typical picture showed abundant debris from damaged mitoses and with the chromosomal material fused to droplet form. Swollen cytoplasm and swollen nuclei dominated the picture. No active mitoses could be found. The combination of 4×10^{-5} *M* Synkavit plus X rays gave a similar picture. The colonies that had been pretreated for 51 days with 4×10^{-6} and 4×10^{-7} Synkavit and then irradiated with 2700 r did not show as much degeneration as those irradiated (FIGURES 3 and 4). The combination 4×10^{-6} plus 2700 r

produced a few mitoses with a somewhat organized appearance. Few mitoses could be found in the combination 4×10^{-7} and 2700 r. They appeared capable of completing division. In general the colonies were in better condition if pretreated with the tolerated doses of Synkavit than those without treatment and then irradiated. There is less degeneration and less contraction throughout all the colonies. Seven days later the differences between the pretreated cultures and those that had irradiation only were quite distinct. The 2700 r irradiation had produced many rounded cells of various sizes, and some of them seemed to be in active mitosis. Multinucleated and micronucleated giant

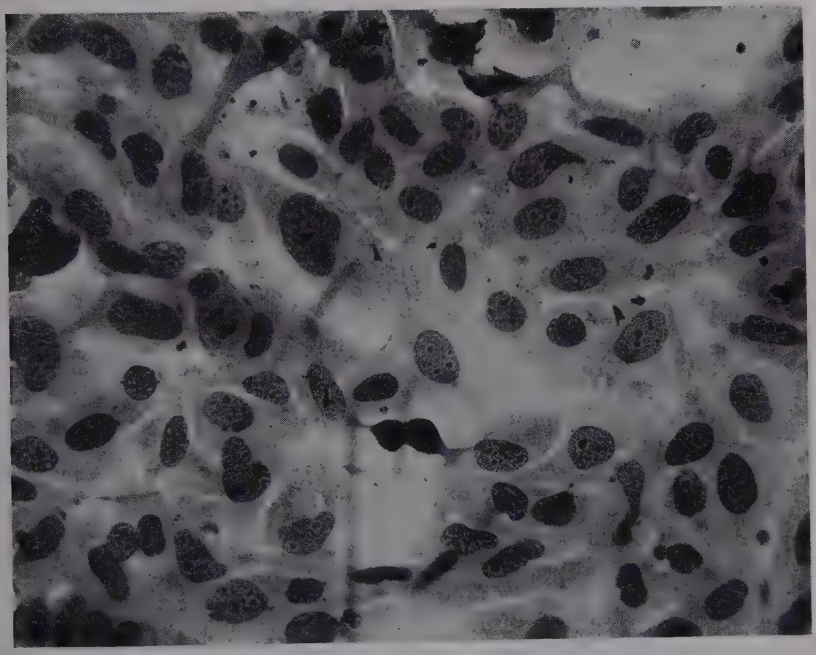


FIGURE 3. Human epidermoid carcinoma strain. Control culture. HeLa. Mayer's hemalum. ×500.

cells and exploding cells were frequent. The combination $4 \times 10^{-5} + 2700$ *r* showed cells well spread out in contrast to the slightly contracted cells treated only with 2700 r. Some areas showed a marked accumulation of arrested mitoses mixed with obviously active, young mitotic figures. Multinucleated and micronucleated cells were increased. These cultures seemed to be in a better state of preservation than those receiving 2700 r alone. Decreasing concentrations of the Synkavit in combination with irradiation showed inversely proportional degrees of protection. These results could be seen in the amount of cell debris and the appearance of intact mitotic stages.

Effects of Synkavit on A.Fi. The A.Fi. tumor cells, which were treated with A Synkavit at the concentration 4×10^{-5} *M*, 4×10^{-6} *M*, 4×10^{-7} *M*, and 4×10^{-8} *M*, showed similar results, but appeared to tolerate higher concentrations.

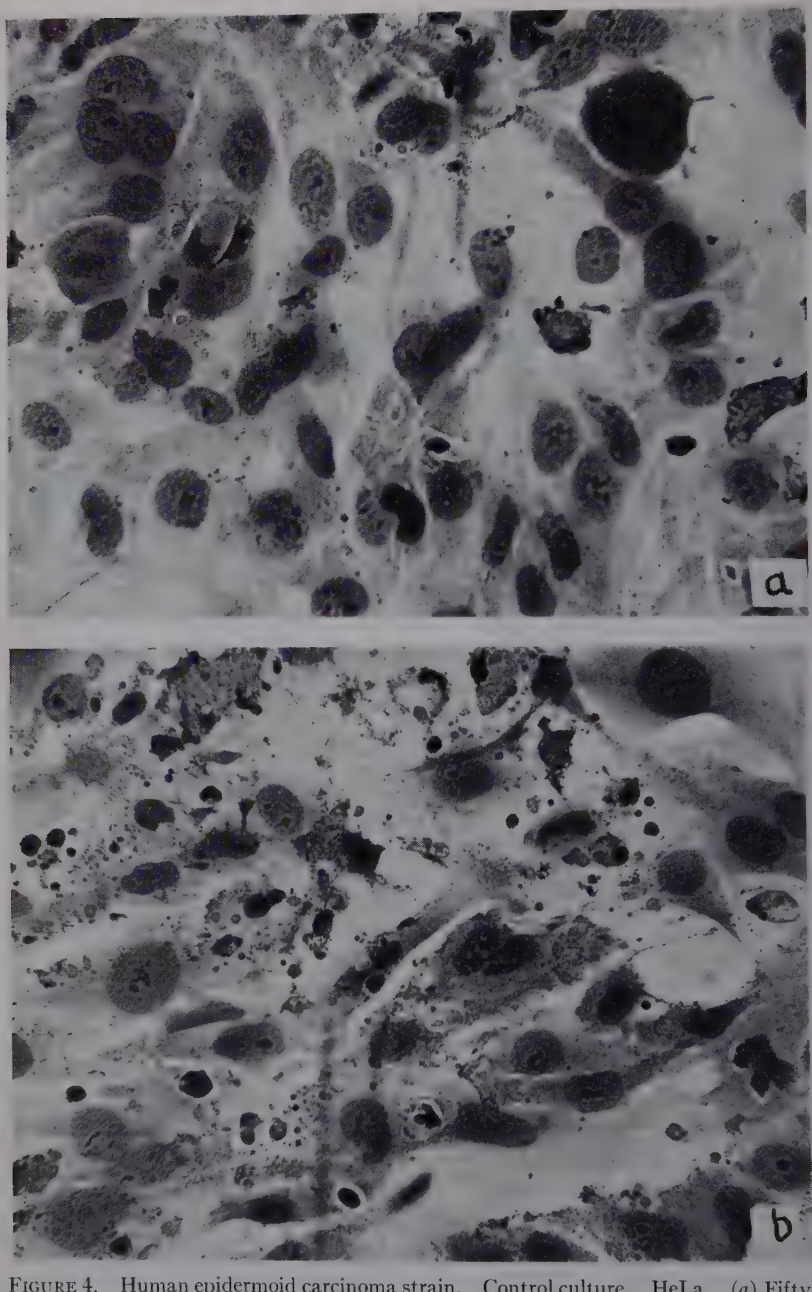


FIGURE 4. Human epidermoid carcinoma strain. Control culture. HeLa. (*a*) Fifty-one days continuous treatment with Synkavit 4×10^{-7} *M* and 30 hours following 2700 r X rays; and (*b*) thirty hours following 2700 r X rays. Note the great difference between this and those in (*a*) with pretreatment with Synkavit. Mayer's hemalum. ×500.

Some Results at 300 r and 900 r

Six hours following 300 r X rays. Direct observations of the living cells at this time and of the stained preparations showed less than 1 per cent of the cells in mitosis. All of them were in the metaphase, and the chromosomes were thick and swollen. A few cells of this type appeared able to complete a cell division (FIGURE 5*a*). The resting cells did not show remarkable changes in the cytoplasm or in the nucleus. Since A.Fi. cultures show giant cells in the controls, a careful comparison was made, yet no significant change in the number of giant cells was found at 6 hours. Also there were no remarkable signs of injury over and above the metaphase arrest.

Twenty-four hours following 300 r X rays. Twenty-four hours following the irradiation a moderate number of mitoses were present (FIGURE 5*b*). Approximately 90 per cent of them were in metaphase, a few in prophase, anaphase, and telophase. Most of these dividing cells showed evidence of chromosomal injury. Some chromosomes appeared swollen, clumped, and others disoriented and broken.[35,37-49] An examination of the multinucleated giant cells[50-53] showed some increase in numbers, thus giving evidence that some of the dividing cells exposed during radiation carried through to this stage. The cytoplasm shows some beginning vacuolization. No remarkable changes were found in the resting nuclei.

The living cell observations at this period showed a slight granularity of the cytoplasm as compared to the controls. The response to the irradiation was also reflected in the moderate increase in cell volume. A few degenerating cells were seen.

Six days following 300 r X rays. The earliest observations, made at 6 and 24 hours, represent to some extent the initial phases of cell response to radiation. The cultures available for this study permitted only a limited number of additional observations. These were therefore made day by day, but with data only on stained cultures at 6 days and with protracted observations on living cultures until the cultures petered out.

Between the 2nd and the 6th day the living cultures showed a definite retardation of growth compared to the controls. Degenerating cells increased with time. The early granular appearance of the cytoplasm progressed to the production of markedly granular and vacuolated cells. Even the remaining cells in mitosis (FIGURE 5*c*) showed such changes in the cytoplasm. The accumulation of mitotic figures increased steadily and, in the stained preparations 6 days following irradiation, 8 to 10 per cent of all the cells were in metaphase. Also there were present a few prophases, anaphases, and telophases. The fixing and staining procedure carried with it the hazard of eliminating the cells that had withdrawn their processes in the more rounded mitotic phases. This obviously prohibited getting exact data from the stained preparations. The mitotic cells in the stained preparations showed clumped and swollen chromosomes, and many of the metaphases showed chromatin that had degenerated to darkly stained small droplets (FIGURE 5*b*). Some of the metaphases not so involved had still intact spindle substance. Obvious telophases, but greatly damaged, showed clumped, disorganized chromosomal material in both daughter cells and with distinct chromosome bridges. As in the 24-hour

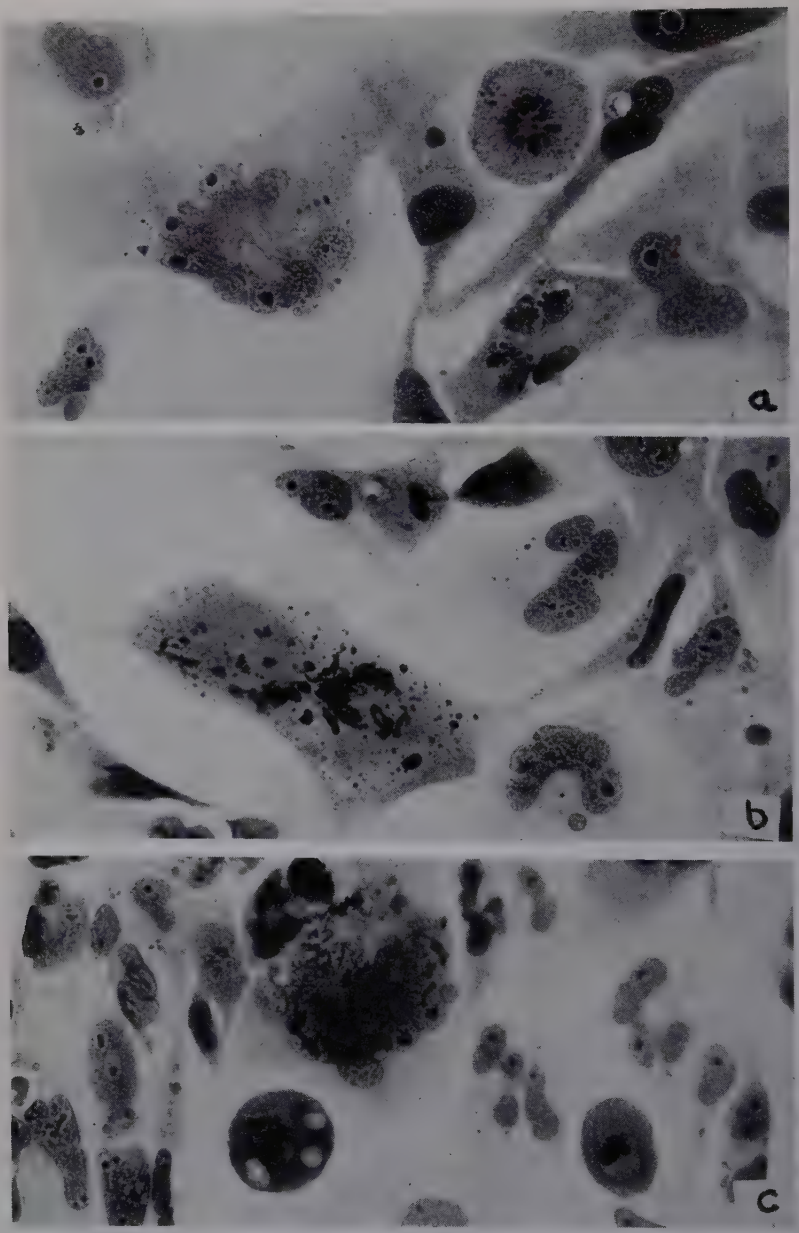


FIGURE 5. Human fibrosarcoma strain A.Fi. (*a*) Six hours following 300 r; injured metaphase with radial pattern of the cytoplasm and similarly dispersed chromosome fragments; injured nuclei; (*b*) twenty-four hours following 300 r X rays; giant cell arrested during metaphase movement; telophase with chromosome bridge and fused chromatin in both daughter cells; and (*c*) six days following 300 r X rays; two injured metaphases; large one shows cysts in the cytoplasm; giant cell with cluster of nuclei and some micronuclei. Mayer's hemalum. ×500.

observations, there was an accumulation of multinucleated giant cells, but at this time in greater numbers and with more exaggerated forms. In fact there was a definite increase in multinucleated cells with micronuclei. Many of the nuclei in general showed folds and cysts. There were also present varying stages of phagocytosis of degenerating cells.

The outcome of this irradiation was followed in order to get evidence of final destruction or eventual recovery. Eight days following irradiation an increasing contraction and reduction of the central areas of the colonies were apparent and with a decreasing population due to loss of cells in the zone of outgrowth. In some areas giant cells and apparently inactive mitotic phases persisted for days. The observations were continued for two weeks following the radiation at which time only the tiniest destroyed remnants remained.

Six hours following 900 r X rays. Six hours following irradiation with 900 r the living A.Fi. cultures showed definite swelling of the cytoplasm and also showed a somewhat granular appearance. No active mitosis could be found. However, a moderate number of rounded, apparently degenerated cells was found scattered over the active expanded cells of the colony. Some obvious mitotic figures with smudged chromatin were present (FIGURE 6*a*). In their rounded form, it was not always possible to distinguish the number of injured dividing cells. The cytoplasmic swelling process was even reflected in the thinned and stretched-out internuclear bridges. There was evidently an exaggeration of the pinching-off process, quite typical for the A.Fi. strain. In the irradiated cells the expansion of the cytoplasm may be sufficiently great to break the nuclear bridges, thus leading to the production of multinucleated cells containing micronuclei of varying sizes. Frequently these stain very poorly and many of them contain no nucleolar material. Elsewhere, the general picture is that of nuclear hypertrophy. The indented and bent nuclei, typical for the control picture of A.Fi., are not to be found and appear to be replaced by more swollen and less elastic nuclei.

The increase in multinucleated and micronucleated cells in the early periods following irradiation suggests an origin from late-polyploid mitotic stages that were damaged during irradiation followed by what appears to be a disorganized reconstruction effort and without a regular spindle or attempt at cleavage. These 6-hour, postirradiation cultures show definite injury in several categories: mitotic arrest; swelling of all components of the cytoplasm and nuclei; production of multi- and micronucleated cells, and demonstrable changes in nucleolar morphology.[28-31]

Twenty-four hours following 900 r X rays. This period following irradiation showed in the living cells a definite increase in the cytoplasmic damage (FIGURE 6*b*). Granules and vacuoles in the cytoplasm were noted with great cellular swelling. No mitotic figures were found. There was, however, a moderate number of degenerating and dead rounded cells.

In the stained preparations, we saw a number of old, interrupted mitotic cells whose chromosomal material was found as beaded dark bodies, generally at the cell periphery. No prophases or other mitotic stages were recognizable. The cytoplasm of the resting cells was distended with multilocular cysts. The number of cells with large single nuclei was distinctly increased. Other cells showed darkly staining shrunken nuclei pressed against the cellular membrane

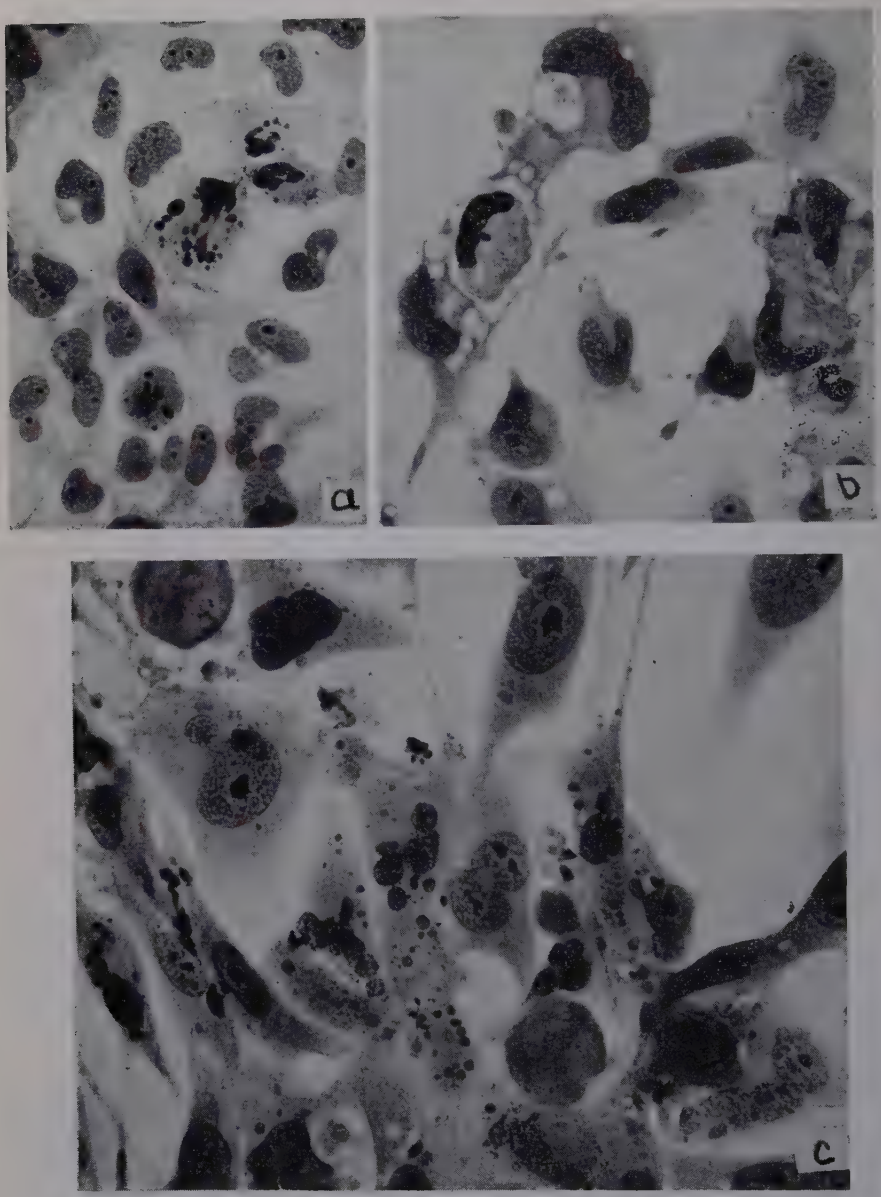


FIGURE 6. Human fibrosarcoma strain A.Fi. (*a*) Six hours following 900 r X rays; large tripolar telophase with dispersed chromatin droplets; arrested damaged metaphase; (*b*) twenty-four hours following 900 r X rays; degenerating mitotic cell surrounded by greatly vacuolated cells; and (*c*) six days following 900 r X rays; two metaphases present; cells enlarged; many of them show multi- and micronucleated condition. Mayer's hemalum. ×500.

by the cystic cytoplasm. A variety of cells showed nuclei with long internuclear bridges but they were less in number compared to the 6-hour cultures. The nucleoli were generally retracted. In comparison with the 6-hour cultures, the results of this irradiation showed first of all not active mitotic figures but many rounded, degenerating, and dead cells. The surviving cells showed greater injury in several categories, both nuclear and cytoplasmic, with increase in multinucleated cells. These confirm our earlier findings with strain A.Fi. where giant cell production was a prominent feature of the radiation responses.

Six days following 900 r X rays. Up to this time between the 2nd and 6th day, a definite arrest of outgrowth occurred. Most noticeable was the appearance of rounded cells that increased progressively with time and were found scattered over a background of well-expanded thin cells. During this preliminary period (2 to 6 days), there was a progressive accumulation of these rounded and degenerating cells, some of which could be clearly seen in mitosis. In addition to the accumulation of rounded cells, there was a definite change in the cytoplasm of the expanded background cells. These appeared progressively more vacuolated with time and also showed enlargement of the nuclei, many of which showed refractile bright nucleoli.

The stained preparations of the 6th day also showed the marked damage throughout the colonies previously followed progressively in the living cultures (FIGURE 6*c*). The cytoplasm and the nucleus were considerably increased in volume with many multilocular cysts in the cytoplasm and great variations in the nuclei from highly polyploid types with one to several nuclei to greatly multinucleated forms, some containing 100 or more micronuclei. A few of the typical pinched-off nuclei with persisting intercellular bridges were still present. There were also very few of the original indented nuclei in the swollen cells. Some resting cells showed densely stained contracted nuclei at the periphery of the cell near the cell membrane. All stages of mitosis could be found but with marked arrest and predominance of metaphases. The other phases found included a few regular prophases and some damaged anaphases and telophases. These and the metaphases showed clumped and greatly disorganized chromatin and chromosomal fragments scattered throughout the cytoplasm.

Because of the progressive degeneration, further treatment of these cultures presented a problem. Some were transferred and others were given fluid renewals without further disturbance. In both cases there was the same progressive destruction beyond the 6th day and up to about 14 days when the cultures disintegrated. A much longer survival in plasmatic media was obtained in our early studies and, apparently, is related to the better nutritional quality of cells in these media.[32-34,36]

Some Observations Following 1000 r and 2000 r

Responses to 1000 r. A group of roller tubes, each containing only 4 colonies growing directly on the tube wall and no tube slips with colonies, were followed progressively in the living state before and after the irradiation. Prior to radiation the A.Fi. colonies showed the usual thin discoidal pattern of healthy, almost transparent colonies, containing well-expanded cells and numerous cells in mitosis. Twenty-four hours following irradiation the colonies were some-

what retracted with rounded degenerating cells scattered in all fields. The number of mitoses was definitely reduced. Some vacuolization of the cytoplasm was already evident.

Observations beyond 24 hours were carried out from day to day. The picture was that of a progressive destruction of cells and a darkening of the central area of the colony. Mitotic figures at first appeared to accumulate but soon were apparently at a standstill and eventually rounded up and washed away or disintegrated, leaving only cell debris. This picture may be compared with the grosser cytopathogenic responses seen in virus destruction, especially as regards the necrotic phases. Accompanying this severe destruction, there was a phase of regeneration, since new active mitoses appeared among the background cells. These were in general greatly expanded and often vacuolated and with granular accumulations. The nucleoli of the background cells appeared contracted and quite refractile.

Continued observation demonstrated the persistence of the arrested mitotic stages up to the end of the first week. By the end of 3 weeks only a few old, rounded, rather clear mitotic cells persisted. These were found among a ring of degenerating and dead cells and cell debris. Some of the greatly swollen cystic micro- and multinucleated giant cells were still present at 3 weeks. These gradually disappeared during the remaining period of observation, that is, until the 28th day when the colonies died out.

Responses to 2000 r. In a similar comparative series in the same media the responses to 2000 r were followed. The general pattern of the cellular response and the colony destruction was much like that following 1000 r. There was, however, more intense destruction and a general shortening of the total survival period. In all other respects the cellular responses appeared somewhat similar.

These particular experiments, carried out in fluid media with cells grown directly on glass, appear to have a rather limited survival. The previous experiments carried out many years before (1939 to 1940) with the A. Fi. strain, but in plasmatic media containing embryo extract, demonstrated longer survival. These experiments have not defined the maximal dose tolerated for a given set of conditions but provide some interesting aspects of the cellular responses and evidence of the limited survival pattern at certain dosages.

Some Results at 2700 and 8100 r

Six-hour observations following 2700 r. Exposure to 2700 r resulted in the production of many exploding cells of Canti (FIGURE 7*a*). In these and other severely damaged mitotic cells, numerous, densely stained chromatin droplets were found especially in the central area of the colonies. Some of these degenerating cells were still recognizable as damaged metaphases or telophases whose chromatin appeared as fused, smudged, or stringy masses through the cytoplasm. No active, healthy mitotic cells were found.

The resting cells were markedly increased in volume. Their cytoplasm showed numerous vacuoles. The nuclei were also swollen, and their staining affinity was considerably reduced. The nucleoli often showed a slight halo.

Six-hour observations following 8100 r. Cursory observations demonstrated readily the extensive degeneration at this early period. In the most extreme fields of damage, degenerating cells were found and scattered over the active

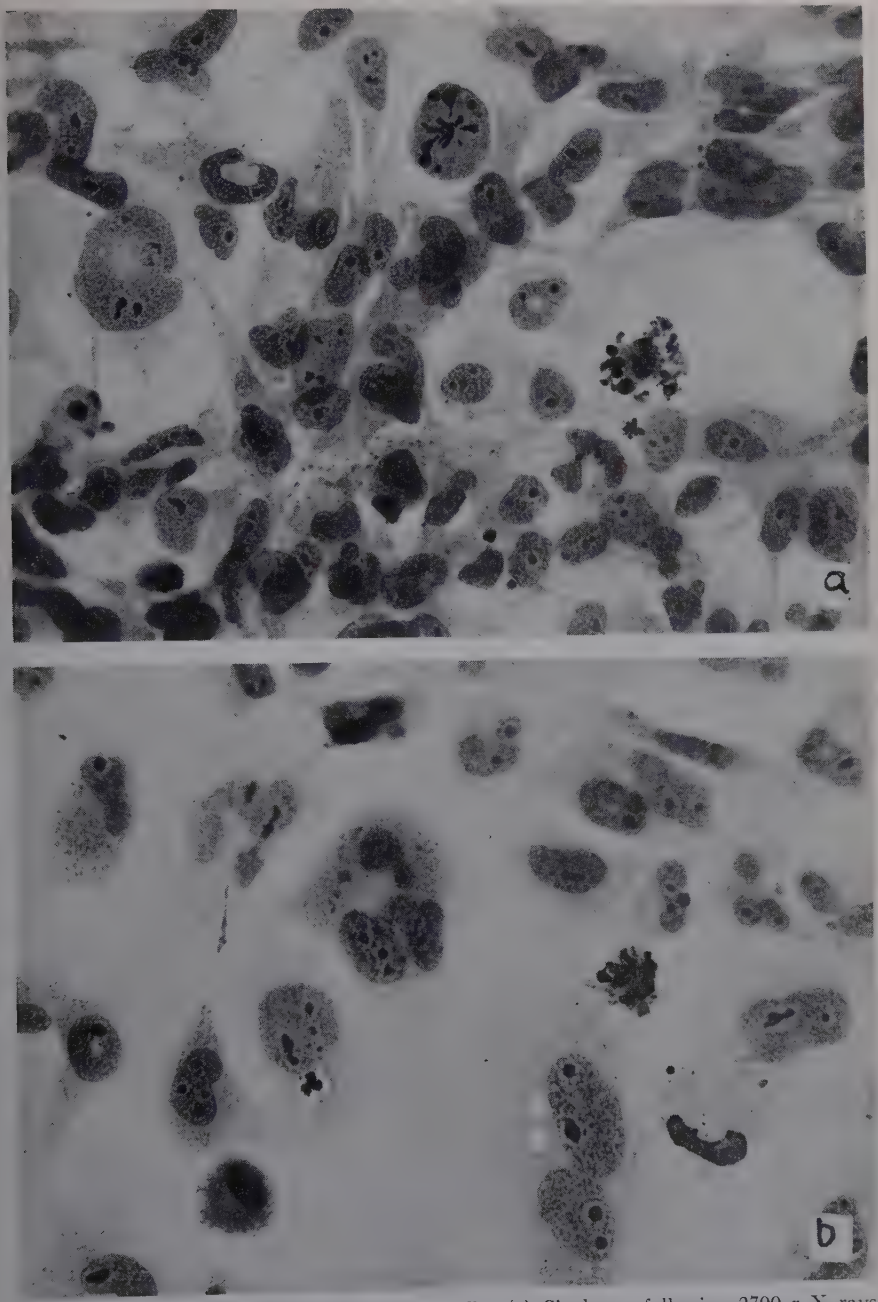


FIGURE 7. Human fibrosarcoma strain A.Fi. (*a*) Six hours following 2700 r X rays; early damage shown by exploding cell and metaphase cell with fused chromosomal material; and (*b*) six hours following 8100 r X rays; severe early damage of 3 degenerating cells, 2 of exploding type. Mayer's hemalum. ×500.

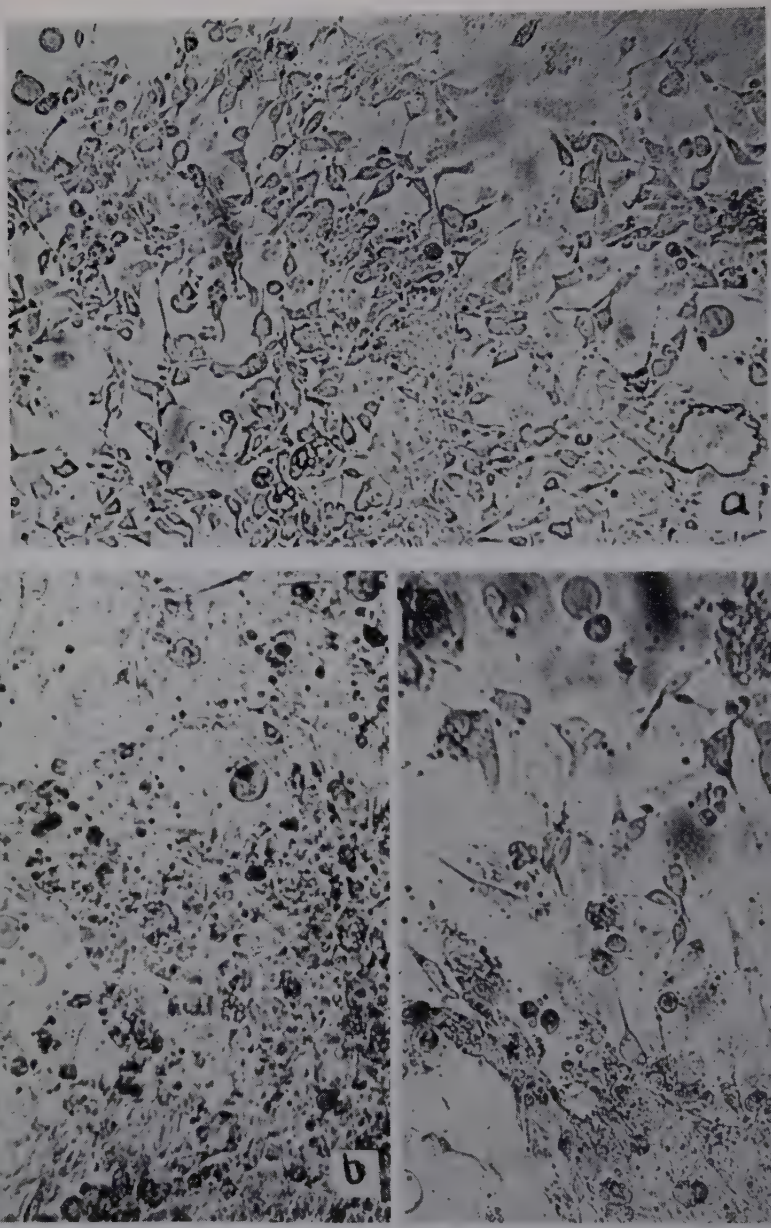
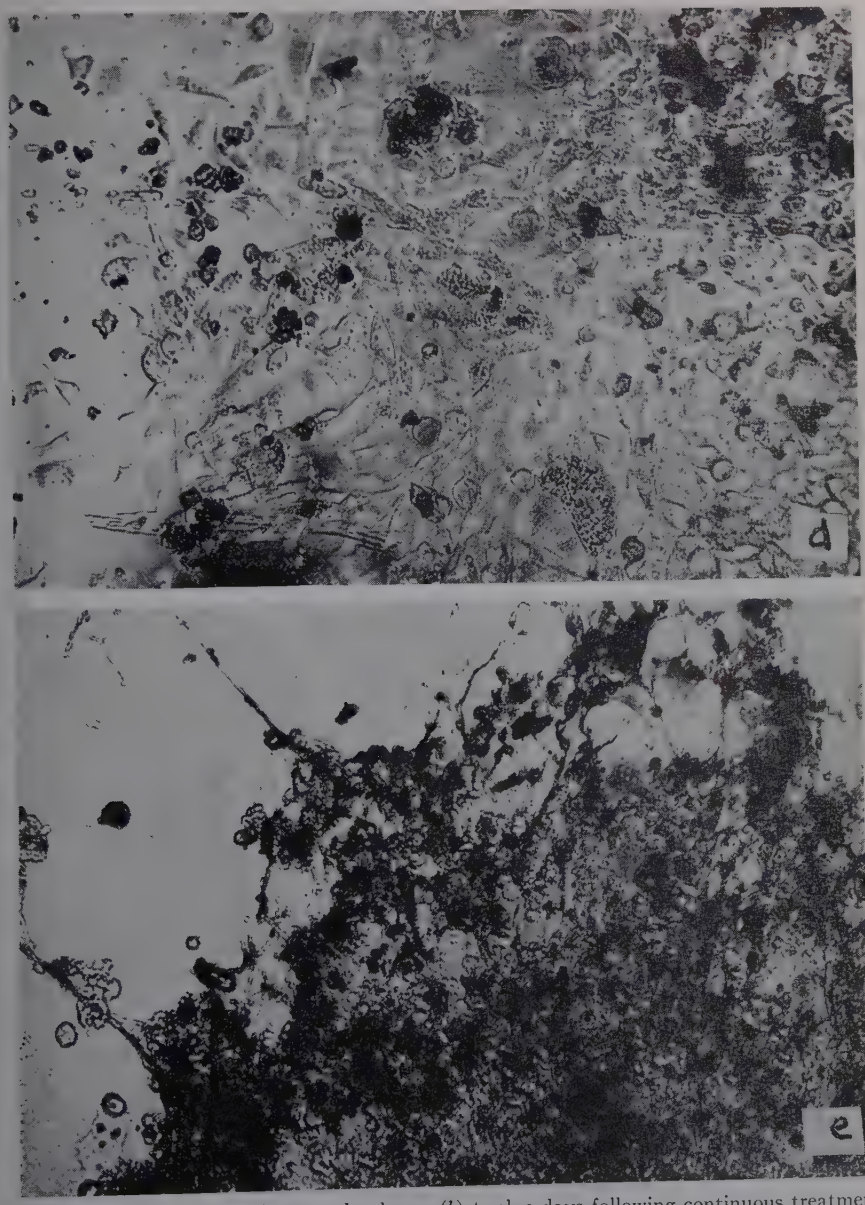


FIGURE 8. Observation of living roller-tube cultures of strain A.Fi. following Synkavit with Synkavit 4×10^{-6} *M* and 11 days following 1000 r X rays; the cultures in this series debris than those without Synkavit; (*c*) eleven days following 1000 r X rays; culture shows some mitotic stages; (*d*) nineteen days following continuous treatment with 4×10^{-6} *M* these cultures contain many active cells and numerous, possibly inhibited dividing cells; and cells almost completely covered with dark masses of dead cells and cell debris.



and X-ray treatment. (*a*) Control culture; (*b*) twelve days following continuous treatment with Synkavit were richer in cells, with fewer degenerating and dead cells, and with less degenerating cells, dead cells, and cell debris lying over a background of fairly clear cells; Synkavit and 18 days following 1000 r X rays; by comparison with (*c*) it may be noted that (*e*) nineteen days following 1000 r X rays; markedly contracted colony shows some background

area in varying numbers through the colony. The cellular damage produced considerable contraction and restriction of the outgrowth with progressive loss of cells (FIGURE 7*b*). No active or normal-appearing mitoses could be found. This was due to the fact that the more sensitive mitotic stages were rapidly destroyed and no progression of mitosis appeared. The surviving background of apparently resting or interphase cells was greatly swollen, and many showed extensive multilocular vacuolization. The damage is also manifested in the swollen nuclei that presented a somewhat homogenous and foamy appearance in the stained preparations. The controls by comparison showed a more or less distinct granular appearance. The severe damage seen in the cultures irradiated at 2700 r and 8100 r did not necessitate extended observations beyond 6 hours. There is only the remotest likelihood that any of these cells could have been recovered.

Effects of Synkavit and X rays on A.Fi. For our studies on possible radiosensitization of cells in cultures, we used basically the experimental arrangement suggested by J. S. Mitchell and I. Simon-Reuss. To cover several variations, we set up the following experimental groups of roller tubes, each with 4 colonies on tube slips. These were exposed for 18 hours to the following concentrations of Synkavit: 4×10^{-5} *M*, 4×10^{-6} *M*, and 4×10^{-7} *M*. Suitable controls were also maintained. Each group was then subdivided in 3 parts. One group was irradiated with 300 r, 1 group with 900 r, and 1 group received no radiation. Groups of controls not receiving Synkavit were irradiated identically, and all tube slips were fixed 6 hours following irradiation and 24 hours following the administration of Synkavit.

In the same way, we irradiated groups with 300 r and 900 r after a 6-hour period of Synkavit administration. These cultures were fixed 24 hours following irradiation and 30 hours after Synkavit treatment. Another group from this experiment was carried on for 6 days.

The cultures irradiated with 300 r and 900 r after previous treatment with Synkavit showed in general the same cytological changes as the nonpretreated ones at the 6-hour period. The cultures receiving 300 r showed few mitotic figures. They were preserved better in the Synkavit plus X-ray group, whereas the cytoplasm showed more damage. Some cells in both groups showed phagocytosis of the damaged mitotic cells.

At 24 hours following irradiation the arrested mitotic figures showed more degenerative forms in the irradiated cultures than in the Synkavit pretreated ones, a fact that was confirmed by the estimation of colony size at the 6th day. At this time the irradiated colonies were one-half of the control-colony size and the pretreated ones were about two-thirds of the size of the control cultures. By comparison, the same pattern of results was obtained under irradiation with 900 r. Here too the mitotic figures showed a definitely better preservation under pretreatment with Synkavit. This, however, did not exclude an increase of multinucleated and micronucleated cells in all preparations of the combined treatment. On the basis of these observations we may assume that the damage to the chromosomes in the pretreated culture was heavy enough to cause a disorganization of the telophase stages.

These experiments were continued until all the colonies disintegrated during

the course of two to three weeks following irradiation. The final survival time was the same in the two types of treatment.

Irradiation (1000 r and 2000 r) combined with 4×10^{-6} M Synkavit. This experiment at a higher irradiation level showed strikingly different results during the early periods following treatment. X rays (1000 r) administered to cultures treated 24 hours previously with 4×10^{-6} *M* Synkavit showed in the living culture definitely less destruction than the cultures not receiving pretreatment with Synkavit. Photomicrographs taken on the 11th and the 18th day demonstrate better attachment of the cells to the glass and more mitotic activity (FIGURE 8*a* to *e*). Although both experimental groups disintegrated at about the same time, there was at least no evidence for a radiosensitization.

The dosage of 2000 r was obviously too high to permit good comparisons since early destruction was severe.

Discussion

We have tried to evaluate the effects of Synkavit A and B on the radiation responses of A.Fi. and HeLa cells. These experiments were somewhat similar to those done on some fibroblasts,[4,5] which demonstrated an increased radiosensitivity under Synkavit treatment under the conditions of the experiments reported here. However our human tumor strains A.Fi. and HeLa did not demonstrate such an effect. Both of these strains tolerated the combination of Synkavit and X rays to a higher degree than the exposure to X ray alone. There was also some evidence of protection of the mitotic stages by Synkavit. These results are similar to those of Strangeways and Fell,[49] who kept chicken fibroblasts at 0° to 5° C. for 5 to 24 hours immediately following irradiation with evidence of moderation of X-ray damage. They may be due to a partial suppression of the mitotic activity caused by the Synkavit.

Summary

Two human cell strains, the adenocarcinoma HeLa and the fibrosarcoma A.Fi. have been used for tests on the possible radiosensitizing capacities of the synthetic vitamin K Synkavit. Concentrations of 4×10^{-6} and 4×10^{-7} have been tolerated by the human-tumor cells. No remarkable enhancement of radiation effect could be obtained in these studies. The tumor tissue pretreated with Synkavit showed less destruction following irradiation with X rays than after irradiation without Synkavit treatment. However, a final protection was not detectable. The responses of the A.Fi. sarcoma cells to irradiation of various doses is described.

Acknowledgments

We are indebted to R. J. Dickson and R. F. Plott, Department of Radiology, for their kind help with the radiation procedures.

References

1. MITCHELL, J. S. 1948. Clinical trials of tetrasodium 2-methyl-1:4-naphthohydroquinone diphosphate, in conjunction with X-ray therapy. Brit. J. Cancer. **2:** 331.
2. MITCHELL, J. S. 1953. Clinical assessment of tetra-sodium 2-methyl-1:4-naphtho-

hydroquinone diphosphate as a radiosensitizer in the radiotherapy of malignant tumours. Brit. J. Cancer. **7:** 313.
3. Mitchell, J. S. 1954. Clinical trials of tetrasodium 2-methyl-1:4-naphthohydroquinone diphosphate as a radiosensitizer. British Empire Cancer Campaign Annual Report.
4. Friedmann, E., D. H. Marrian & I. Simon-Reuss. 1952. Mitosis of chick fibroblasts in the presence of unsaturated imides and sulphydryl compounds. Biochim. et Biophys. Acta. **9:** 61.
5. Friedmann, E., D. H. Marrian & I. Simon-Reuss. 1952. Halogen derivatives of the 1:4-naphthoquine group and the maleic acid series in their action on the mitosis of chick fibroblasts. Biochim. et Biophys. Acta. **8:** 680.
6. Friedmann, E., D. H. Marrian & I. Simon-Reuss. 1948. Analysis of antimitotic action of certain quinones. Brit. J. Pharmacol. and Chemother. **3:** 263.
7. Hughes, A. & I. Simon-Reuss. 1953. The immediate effect of some phosphorylated naphthohydroquinones on dividing cells. Brit. J. Cancer. **7:** 142.
8. Gey, G. O. & F. B. Bang. 1951. Viruses and cells. A study in tissue culture applications. I. Cells involved—availability and susceptibility. Trans. N.Y. Acad. Sci. **14:** 15.
9. Scherer, W. F., J. T. Syverton & G. O. Gey. 1953. Studies on the propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J. Exptl. Med. **97:** 695.
10. Gey, G. O. & F. B. Bang. 1939. Experimental studies on the cultural infectivity of lymphopathia venerea virus maintained in tissue culture. Bull. Johns Hopkins Hosp. **65:** 393.
11. Gey, G. O., F. B. Bang & M. K. Gey. 1954. Responses of a variety of normal and malignant cells to continuous cultivation and some practical applications of these responses to problems in the biology of disease. Ann. N.Y. Acad. Sci. **58**(7): 976.
12. Gey, G. O., M. K. Gey & W. F. Fitzwilliams. 1935. Viability of strains of human and rat normal and malignant cells maintained in culture following exposures to varying doses of X ray. The International Cancer Research Foundation. Report of Activities, Philadelphia, Pa. : 35.
13. Gey, G. O. 1954–1955. Some aspects of the constitution and behavior of normal and malignant cells maintained in continuous culture. The Harvey Lectures. : 154. Academic Press, Inc. New York, N.Y.
14. Gey, G. O. 1954. Discussion on cellular structure. Trans. 15th Josiah Macy Jr. Conf. on the Problems of Aging. : 39.
15. Gey, G. O. & M. K. Gey. 1936. The maintenance of human normal cells and human tumor cells in continuous culture. I. Preliminary report: cultivation of mesoplastic tumors and normal tissue and notes on methods of cultivation. Am. J. Cancer. **27:** 45.
16. Gey, G. O., P. Shapras, F. B. Bang & M. K. Gey. 1955. Some relations of inclusion droplets (pinocytosis—Lewis) and mitochondrial behavior in normal and malignant cells. Symposium on fine structure of cells. : 38. E. P. Noordhoff, Ltd. Groningen, The Netherlands.
17. Stoler, M. & M. K. Gey. 1953. Destruction of human fibrosarcoma strain (A.Fi.) in tissue cultures following the growth of poliomyelitis viruses. Bull. Johns Hopkins Hosp. **92:** 385.
18. Owens, O. v. H., G. O. Gey & M. K. Gey. 1953. A new method for the cultivation of mammalian cells suspended in agitated fluid medium. Am. Assoc. Cancer Research Proc. **1:** 41.
19. Owens, O. v. H., M. K. Gey & G. O. Gey. 1954. Growth of cells in agitated fluid medium. Ann. N. Y. Acad. Sci. **58**(7): 1039.
20. Earle, W. R., J. C. Bryant, E. L. Schilling & V. J. Evans. 1956. Growth of cell suspensions in tissue culture. Ann. N. Y. Acad. Sci. **63**(5): 666.
21. Puck, T. T., P. I. Marcus & S. J. Cieciura. 1956. Clonal growth of *in vitro*. J. Exptl. Med. **103:** 273.
22. Puck, T. T. & P. I. Marcus. 1955. A rapid method for viable cell titration and clone production with HeLa cells in tissue culture. The use of X-irradiated cells to supply conditioning factors. Proc. Nat. Acad. Sci. **41:** 432.
23. Puck, T. T. & P. J. Marcus. 1956. Action of X rays on mammalian cells. J. Exptl. Med. **103:** 653.
24. Hsu, T. C. 1954. Cytological studies on HeLa, a strain of human cervical carcinoma. I. Observations on mitosis and chromosomes. Texas Repts. Biol. and Med. **12:** 833.
25. Hsu, T. C. & P. S. Moorehead. 1956. Chromosome anomalies in human neoplasms with special reference to the mechanisms of polyploidization and aneuploidization in the HeLa strain. Ann. N. Y. Acad. Sci. **63**(6): 1083.
26. Hsu, T. C. & C. M. Pomerat. 1953. Mammalian chromosomes *in vitro*. III. On somatic aneuploidy. J. Morphol. **93:** 301.

27. Hsu, T. C. 1955. Mammalian chromosomes *in vitro*. VI. Observations on mitosis with phase cinematography. J. Nat. Cancer Inst. **16:** 691.
28. Canti, R. G. & F. G. Spear. 1927–1928. The effect of irradiation on cell division on tissue culture *in vitro*. Proc. Roy. Soc. **B102:** 92.
29. Canti, R. G. 1927. The effect of irradiation on tissue. St. Bartholomew's Hosp. Rep. **60:** 57. London, England.
30. Halberstaedter, L., G. Goldhaber & J. Doljanky. 1942. Comparative studies on the radiosensitivity of normal and malignant cells in culture. I. The effect of X rays on cell outgrowth in cultures of normal rat fibroblasts and rat benzpyreneinduced sarcoma. Cancer Research. **2:** 28.
31. Spear, F. G. 1935. Tissue cultures II and III. Its application to radiobiological research. Brit. J. Radiol. **8:** 68, 280.
32. Goldfeder, A. 1940. Further studies on the effects of irradiation on proliferation and metabolic processes of normal and malignant tissues. IV. Effects produced by different dosage rates of X-ray radiation on the proliferation of various tissues grown *in vitro*. Radiology. **35:** 210.
33. Goldfeder, A. 1948. The effects of radiation on mammalian tissue cells, normal and malignant. Cancer Research. **8:** 383.
34. Goldfeder, A. & A. Cameron. 1948. Growth in tissue culture of analogous mouse mammary carcinomas and their response to radiation. Cancer Research. **8:** 465.
35. Friedenwald, J. S. & J. Sigelman. 1953. The influence of ionizing radiation on mitotic activity in the rat's corneal epithelium. Exptl. Cell Research. **4:** 1.
36. Reid, T. R. & M. P. Gifford. 1952. A quantitative study of the effect of X radiation on cells *in vitro*. J. Nat. Cancer Inst. **13:** 431.
37. Reid, T. R. 1943. The effect of X rays on cells cultivated *in vitro*. II. Recovery factor. Brit. J. Radiol. **16:** 61.
38. Reid, T. R. 1943. The response of cells *in vitro* to variation in X-ray dosage. Brit. J. Radiol. **16:** 131.
39. Lasnitzki, J. 1957. Cancer cells in tissue culture. Butterworth and Co. London, England.
40. Hollaender, A. 1956. Modification of radiation response. Bull. of atomic scientists. **12:** 76.
41. Hughes, A. F. W. & H. B. Fell. 1949. Studies on abnormal mitosis induced in chick tissue cultures by mustard gas ($\beta\beta'$-dichlordiethyl sulphide). Quart. J. Microscop. Science. **90:** 37.
42. Zirkle, R. E. & W. Bloom. 1953. Irradiation of parts of individual cells. Science. **117:** 487.
43. Ludford, R. J. 1932. Cytological changes after radiation of malignant growth. Sci. Rep. Imp. Cancer Research Foundation. **10:** 125.
44. Laser, H. & L. Halberstaedter. 1929. Radiosensibilitaet normaler und boesartiger Gewebe *in vitro*. Zeitschr. f. Krebsf. **29:** 411.
45. Brues, A. M., A. N. Stroud & G. Scihla. 1951. Mitotic changes accompanying continuous irradiation. Anat. Records. **109:** 411.
46. Stroud, A. M. & A. M. Brues. 1954. Radiation effects in tissue culture. Texas Repts. Biol. Med. **12:** 931.
47. Stroud, A. M. 1956. The effects of continuous irradiation by Tritium on cells cultivated *in vitro*. Ann. N.Y. Acad. Sci. **67**(2): 11–34.
48. Koller, P. C. 1947. The effect of radiation on the normal and malignant cells in man. Brit. J. Radiol. Suppl. 1.
49. Strangeways, T. S. P. & H. B. Fell. 1927. A study of direct and indirect action of X rays upon the tissues of the embryonic fowl. Proc. Roy. Soc. **B2:** 9. London, England.
50. Pomerat, C. M., S. P. Kent & L. C. Logie. 1957. Irradiation of cells in TC. I. Giant Cell Induction in Strain Cultures Versus Elements from Primary Explants. Zeitschrift f. Zellforschg. **47:** 158–174.
51. Pomerat, C. M., S. P. Kent & L. C. Logie. 1957. Irradiation of cells in tissue culture. II. Cinematographic analyses of cell enlargement and mitotic activity following gamma irradiation at 2000 r and 4000 r. Zeitschrift f. Zellforschg. **47:** 175–197.
52. Pomerat, C. M., L. Hayflick, M. V. Fernandes & S. P. Kent. 1958. Irradiation of cells in tissue culture. III. Measurement of injury by *p*H changes and cytopathology in test-tube cultures. Zeitschr. f. Zellforschg. **47:** 245–250.
53. Harper, J. Y., C. M. Pomerat & S. P. Kent. 1958. Irradiation of cells in tissue culture. IV. The effect of 2000 r and 4000 r of gamma irradiation from a cobalt 60 source on cultures of eye tissues. Zeitschr. f. Zellforschg. **47:** 392–400.

RESPONSE OF NEOPLASTIC CELLS *IN VITRO* TO IONIZING RADIATION*

Anna Goldfeder

Cancer and Radiobiological Research Laboratory, The City of New York Department of Hospitals, and New York University, New York, N.Y.

The application of the tissue-culture technique to studies of the direct effects of ionizing radiation on mammalian neoplastic cells had its inception as far back as 1914, when Wood and Prime exposed animal tumor cells cultured *in vitro* to gamma rays of radium.[1] Another paper on this subject appeared in the same year, in which Price and Mottram[2] described the effects of irradiation on a mouse carcinoma and a rat sarcoma grown in hanging drop cultures *in vitro*. A number of papers dealing with effects of ionizing radiation on various types of animal tumors appeared soon after these earlier publications.[3-5] Although the radiation doses were not specifically determined, due to the unavailability of the necessary methods and physical instrumentation at that time, the earlier investigators nevertheless made fundamental observations of the direct effects of irradiation on cellular dynamics. For example the arrest of mitotic division, particularly in prophase, was observed. The fact that dividing cells completed their division during exposure time and the daughter cells appeared normal during the first few generations but showed abnormalities later was also noted. This indicated that the effects of irradiation may not be noticeable immediately, and that a delayed effect exists. Furthermore chromosomal fragmentation, cell enlargements, and cytoplasmic vacuolization were seen to be constant features of radiation effects on cells. The first detailed account of such phenomena induced by ionizing radiation was presented by Strangeways and Oakley on chick fibroblasts.[6] These authors made reference to previous investigators who made similar observations on irradiated mammalian tissues *in vivo*.[7-9] Thus the tissue-culture method was recognized as a valuable tool for studying cellular dynamics following irradiation without the influence of the whole organism.

A large part of experimental work in the field of radiobiology has been concerned with cultivation of normal avian tissues or monocellular organisms. Comparatively much less data are available from experiments with mammalian tissues, either normal or malignant. Improvement of techniques and the development of appropriate nutritive media in more recent years facilitated the application of the tissue-culture method to more complicated tissues such as mammalian. Furthermore improved optical instruments, such as phase contrast microscopy and cinematography, enable the investigators to study the radiation-induced damage not only of the whole cell but of parts of the cell under strictly controlled conditions.[10] With the advance of perfected tissue-culture techniques, it is now possible to follow the productive ability of a single cell following irradiation.[11]

While the major observations of the earlier investigators were of a qualitative nature, the more recent work is directed toward obtaining data on a

* The work described in this paper was supported in part by grants from the National Cancer Institute, Public Health Service, Bethesda, Md.

quantitative basis. It is not intended to present in this paper a complete historical review of this subject. For more detailed historical data pertaining to the applications of the tissue-culture technique in radiobiological research in general, the interested reader is referred to two comprehensive review articles.[12,13]

The major objective of my investigation is to detect the inherent properties that govern cellular radiosensitivity, with special emphasis on neoplastic cells. A brief account of the observations made is presented here. More stress is placed on the morphological and cytological changes occurring in the irradiated tissue cells as related to their inherent properties than on quantitative estimation.

Material and Methods

Transplantable animal tumors grown in isologous hosts, that is, hosts from which they had originated, served as the basic material for these studies. Normal embryonic tissues (human and animal) such as heart, kidneys, tongue, and liver were employed as controls. Small, thin sections excised with very sharp cataract knives from actively growing tumors *in situ* were employed rather than cells cultivated for some length of time *in vitro*, as commonly used by other investigators. The use of freshly excised tissue particles offers several advantages: (1) they more closely simulate conditions when intact tumors consisting of cell aggregates are irradiated *in vivo*; (2) portions of the irradiated sections of the same tumor can be used for reimplantation into parent hosts, thus permitting a comparison of the cellular changes following irradiation when the tissue is grown under normal environmental conditions with those cultured *in vitro*; (3) it has been noted that cultures of normal tissues cultivated for long periods *in vitro* are significantly changed and, in several instances, become malignant;[14,15] and (4) tumors (animal and human) grown in solid media may retain their structure and even differentiate.[16-20]

In the investigations reported here, mouse mammary tumors of similar and dissimilar morphologic structures were employed. The culture medium consisted of rat or human serum, of mouse- or rat-embryo extract (prepared in Tyrode solution), and of chick plasma. The Maximow double cover slip technique[21] was employed in the majority of the experiments. Experience has shown that the cells emigrating, or growing out from the original explant in the plasma clot, produce loose monolayers or sheets. The cells can easily be identified and studied individually by light microscopy or cinematography.

Radiation procedure. A 200 kv X-ray machine operating at 15 or 20 mAmp. was employed. The X-ray beam was filtered through 0.5 mm. Cu plus 1.0 mm. Al; the HVL equaled 1.1 mm. Cu. The dose rates varied from 250 to 10,000 r/min. in air, depending upon the purpose of the experiment. To avoid any possible effect of irradiation on the nutritive medium, the explants were irradiated as follows. The freshly excised tissue sections were spread on a No. 1 round coverslip that was previously attached to a square mica sheet. This was placed above the hollow ground Maximow slide, the concavity of which contained several strips of moist filter paper in order to prevent drying of the tissues. This procedure was used for the control cultures that were not irradiated. The mica sheet was sealed with paraffin and the

tissue was then irradiated. Following irradiation with specific doses of X rays, the tissue particles were removed, washed in Tyrode solution, and 1 or 2 tissue particles were used for 1 culture. For each experiment, 12 cultures were made of the irradiated tissues and 12 of nonirradiated controls. They were incubated at 37.5° C. The growing cultures were bathed in Tyrode solution every 2nd day and subcultured. The zone of the outgrowth from the explant was examined either on living cells or on whole mounts of fixed cultures on the round coverslips in Carnoy's solution and stained with hematoxylin and eosin.

Criteria. The following served as criteria for evaluating the effects of a given X-ray dose: (1) the number of explants that produced new growth; (2) the latent period (that is, the time elapsing between placing the cultures into the incubator and occurrence of a new outgrowth from the explant); (3) the extent of the new growth; and (4) the cell types and their cytological appearance.

Results

In our earlier studies, embryonic tissues (animal and human) and tissues from 1-day-old mice and rats were compared with malignant tissues in their response to irradiation. The results obtained from several experiments are briefly summarized in TABLE 1. These studies elicited valuable observations. A different response to an equal dose of radiation was noted among the various cell types in the outgrowth of the same tissue explant; specifically when embryonic or 1-day-old kidney explants were irradiated and cultivated *in vitro*, the lymphocytes, leukocytes, macrophages, and fibroblasts were selectively destroyed by radiation dosages, which were gradually increased, while the renal epithelial cells continued to proliferate. More details of this work may be found in the original publications.[22]

Further studies in this laboratory demonstrated that mouse mammary tumors, of similar or dissimilar morphologic structure, differed significantly in their response to an equal dose of radiation. This was manifest in the nuclear and cytoplasmic abnormalities of the cells appearing in the outgrowth of the irradiated explants. FIGURE 1*a* and *b* illustrates the two types of tumors as they appeared in microscopic sections before irradiation. It is apparent that both consist of aggregates of epithelial cells.

Cultures of tumor A. The explants from tumor A produced dense sheets of epithelial cells. Active macrophages were numerous and migrated far in advance of the outgrowing epithelial cells. Some fibroblasts were also seen (FIGURE 2*a*).

Explants of tumor A exposed to 5000 to 15,000 r produced sheets of new cells within a 24-hour incubation period. This growth increased within the next 5 to 9 days. However the epithelial sheets were slightly thinner than those of control cultures (FIGURE 3*a*).

A dose of 20,000 r inhibited the growth of the explants. Only about 50 per cent of the irradiated explants produced sheets of epithelial cells within 48 hours of incubation. The remainder produced new epithelium about 7 days later. No wandering cells were noted in these cultures, indicating their greater radiosensitivity compared with cancer epithelium. Doses of 30,000,

40,000, and 50,000 r extended the latent period from 48 hours to 7 days. On the 7th day all the explants showed some growth. The cell nuclei in this new growth varied in shape and size. A dose of 100,000 r and 110,000 r permitted

TABLE 1

EFFECTS OF X RADIATION UPON THE PROLIFERATION *IN VITRO* OF VARIOUS NORMAL EMBRYONIC TISSUES

Type of tissue	Dose (r)	Number of irradiated explants	Growing explants (%)	Remarks
Kidney (from 1-day-old rat)	40,000	24	100	Both epithelial cells and fibroblastic growth were noted.
	60,000	28	78.6	The new growth of irradiated explants was much smaller and thinner compared to nonirradiated cultures. Only epithelial cells were noted in the outgrowth but no fibroblasts or other cell types.
	90,000	36	9.7	This is an average of two experiments. Only about 3 per cent difference was noted in repeated experiments. There was a significant extension in the latent period; a few cells only were grown out from the explant.
	100,000	28	No growth	No growth occurred in the irradiated cultures, while the control cultures produced excellent growth.
Spleen	40,000	24	64.3	The new outgrowth from the explant was significantly thinner and smaller than that of the controls.
	50,000	24	30.0	Only a very small, thin outgrowth of new cells appeared after 8 days of incubation.
	60,000	24	—	No round cells appeared, but a few fibroblasts were evident.
Sarcoma 180	30,000	25	33.3	A liquefied zone around the explant was noted but above this an array of new cells was seen.
	60,000	24	8.33	The surviving cells in the cultures continued to proliferate in 5 series of subcultures, but disintegrated eventually.

25 per cent of the explants to produce growth approximately 7 days after incubation. Some of the cells in these cultures appeared normal; others had large nuclei and an occasional vacuole in the cytoplasm. Exposure to 150,000 r prevented the explants from producing any new growth.

Cultures of tumor B. FIGURE 4*b* shows a zone of outgrowth from a nonir-

FIGURE 1. (*a*) A microscopic field of a mouse mammary adenocarcinoma; note the aggregates of cells that are separated by fibrous connective tissue stroma; the cell nuclei vary in shape and size, some oval, some round; and (*b*) a microscopic field of a section from a mouse mammary tumor also diagnosed as an adenocarcinoma; note the nests and bands of cells, the empty areas, the palely stained nuclei that also vary in size and shape, and a number of mitotic figures. H and E ×350. Reproduced by permission of *Radiology*.

radiated control-tumor B explant. In the zone of the newly growing cells, macrophages, stroma, and infiltrating cells were observed among the cancer cells. The latter were identified by the size of the nucleus and by the prominent nucleoli. Dividing cells were noted within the explants and in the outgrowing zone. This general pattern was noted in the primary cultures that produced zones of new growth. In the subcultures, fewer infiltrating cells and more cancer cells were noted.

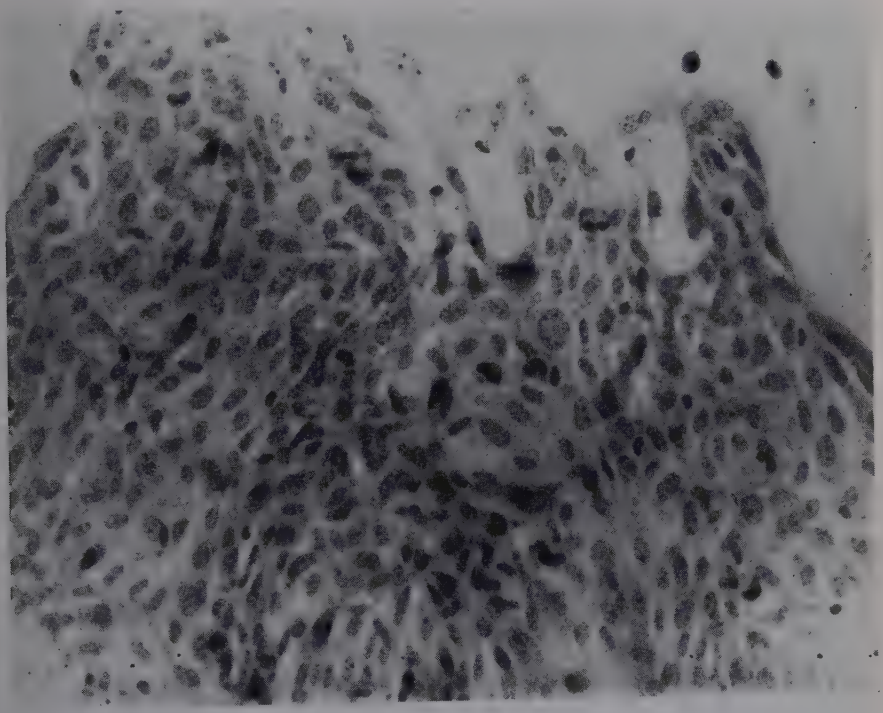


FIGURE 2. An 8-day-old culture of tumor A. Note the luxuriant outgrowth from the explant, the variation in size and shape of the nuclei, and some macrophages.

Contrary to the tumor A, explants of tumor B showed a pronounced effect after exposure to 20,000 r. Thin sheets of the outgrowth consisted of enlarged cell nuclei and vacuolated cytoplasm. Abnormal mitotic figures were also noted in the outgrowth. Examples are presented in FIGURES 5 and 6. With increased X-ray doses, a drastic decrease in the percentage of growing explants occurred, and degenerative cellular changes were more noticeable. Although explants exposed to 50,000 r still produced epithelial sheets, the cells were vacuolated, the nuclei broken up, and the chromatin substance fragmented. A dose of 60,000 r and 80,000 r allowed very small growth in about 25 per cent of the explants. The new growth, however, consisted of loosely arranged single cells instead of sheets. The shape and size of the nuclei differed markedly from those cells grown from nonirradiated explants.

In some areas, only cells with huge nuclei and faintly distinguishable cytoplasm were seen. This is shown in FIGURE 7*b*.

In contrast the outgrowth from the explant tumor A formed sheets of cells containing intact nuclei and nucleoli and homogeneous cytoplasm after a dose of 100,000 r (FIGURE 8). The difference in radiation response between the two epithelial-type tumors is evident. The important observation that evolved from these experiments is the existence of a significant difference in radiosensitivity between tumors of the same morphological classification. On the basis of these results it may be inferred that certain intrinsic factors exist

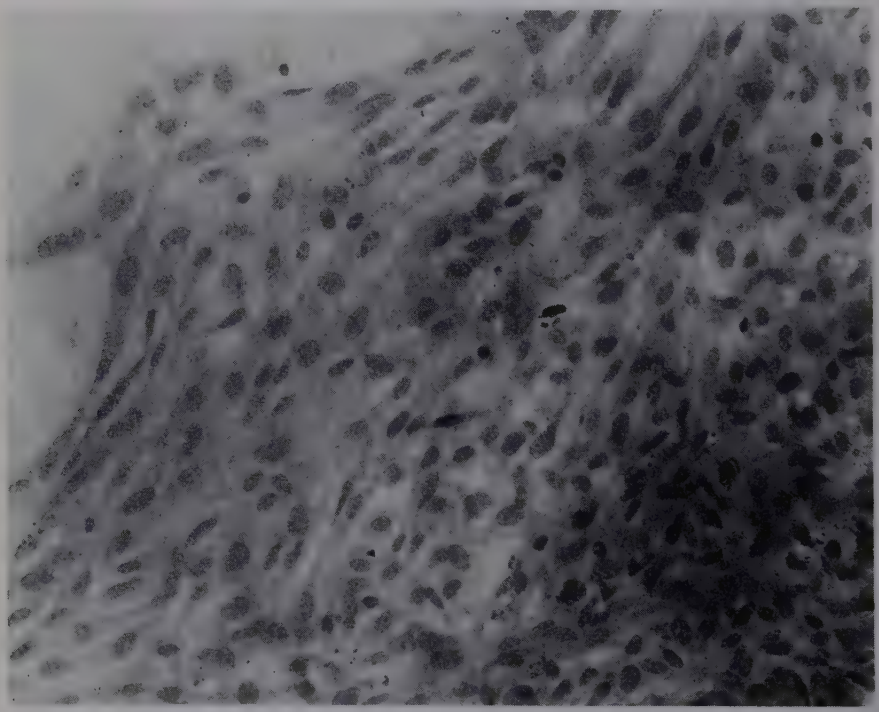


FIGURE 3. A 12-day-old culture of a tumor A explant that was irradiated with a dose of 20,000 r. Note the thinner sheets of the outgrowing cells as compared with FIGURE 2.

that determine radiosensitivity not only among normal but also among tumor cells.

The cytology of two other types of tumors (FIGURE 9*a* and *b*), an epithelial (slower growing, designated C) and a spindle cell (faster growing, designated D), is being studied after irradiation by light and electronmicroscopy at the present time. Both were derived from the mammary gland tissue of the same inbred line and are being carried by serial transplants in isologous parent hosts. They offer therefore an excellent material for studying the inherent properties of the tumor cells as related to radiosensitivity, excluding the possible influence of heterologous host factors. Only a brief summary of the observations made so far is presented.

FIGURE 4. Zone of outgrowth from a nonirradiated control tumor B explant. Note the luxuriant growth of new cells.

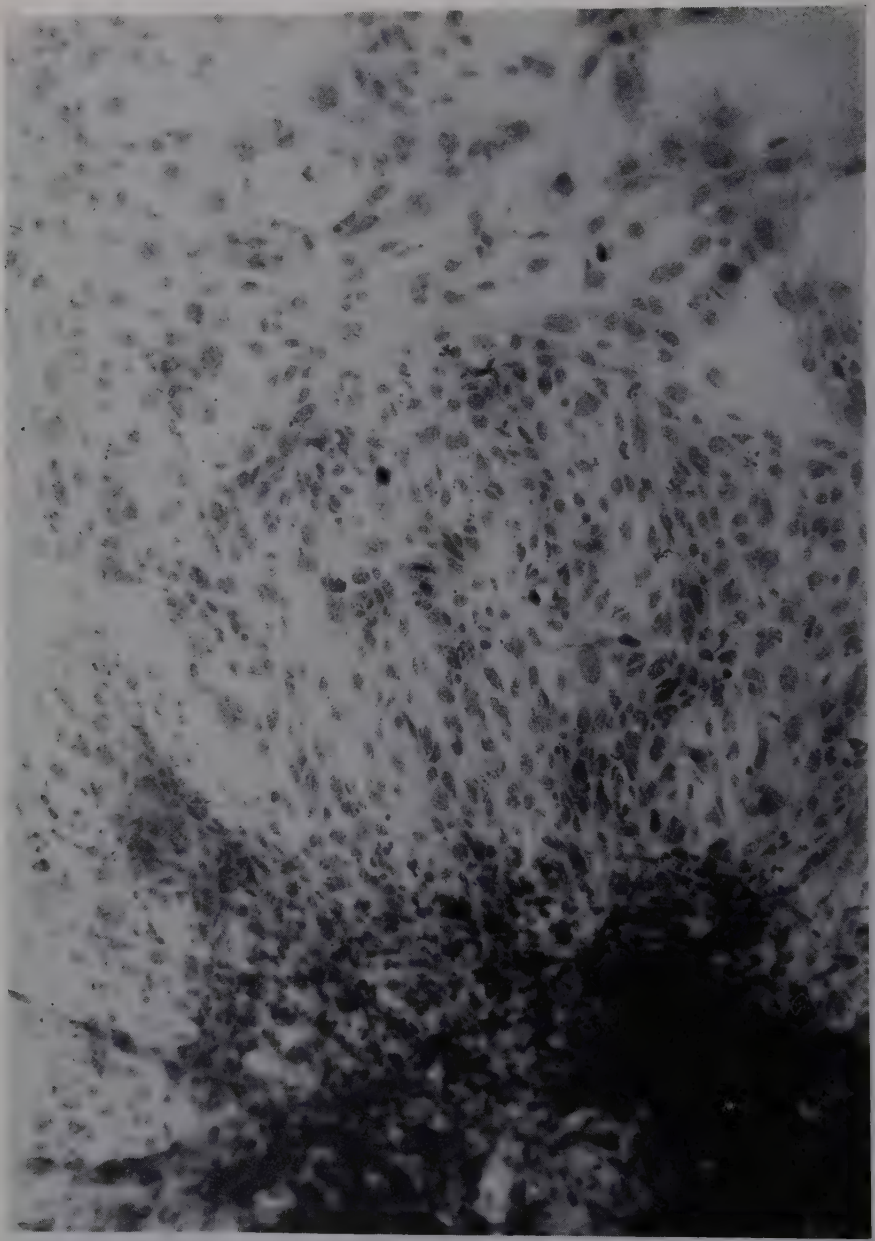


FIGURE 5. Growth from a tumor B explant that was irradiated with 20,000 r. This picture was taken 18 days after irradiation. Note the thin sheets of cells, empty spaces, and the great variation in size of the nuclei, vacuolated cytoplasm, and chromatin particles.

Of particular interest are the dramatic cytological changes occurring in the spindle-cell tumor following irradiation either *in vitro* or *in vivo*. These are unusual abnormal mitotic figures, such as double anaphase figures in the same cell, anaphases with chromosomes lined up at one pole, tripolar, and tetrapolar figures. There are also giant cells that are either multinucleated or mononucleated with large eccentrically located nuclei and vacuoles in the cytoplasm. FIGURES 10, 11, and 12 illustrate examples of these phenomena.

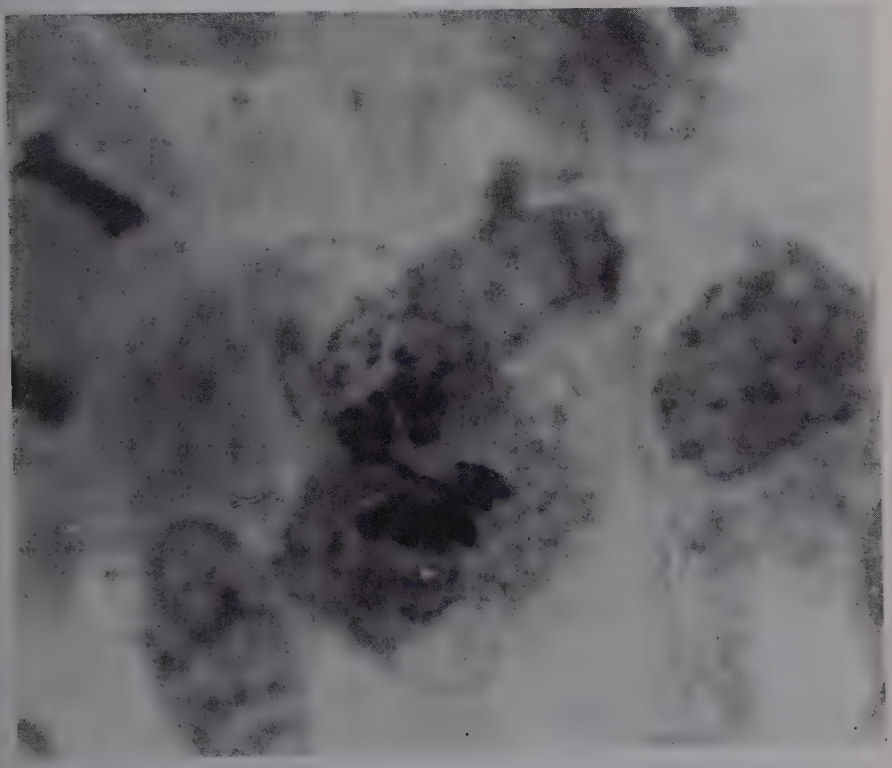


FIGURE 6. An abnormal anaphase with a chromatin bridge and vacuolated cytoplasm (tumor B explant).

No such conspicuous changes were noted in the epithelial tumor C after irradiation with equal doses of X rays. Several questions are raised by the above observations. (1) Why do two types of tumors of the same genetic derivation differ so significantly in their response to irradiation? (2) Are the structural changes, which are the first visible indicators of radiation effects, a consequence of physiological alterations that are not apparent?

To shed light on this problem, metabolic studies on both types of tumors were carried out. The oxygen consumption and aerobic and anaerobic glycolysis were determined *in vitro* by use of the Warburg manometric technique. For the determination of oxidative phosphorylation, the mitochondria

were isolated, and their respiratory and phosphorylative capacity was determined *in vitro*.

The rates of oxygen consumption and aerobic glycolysis were found to be

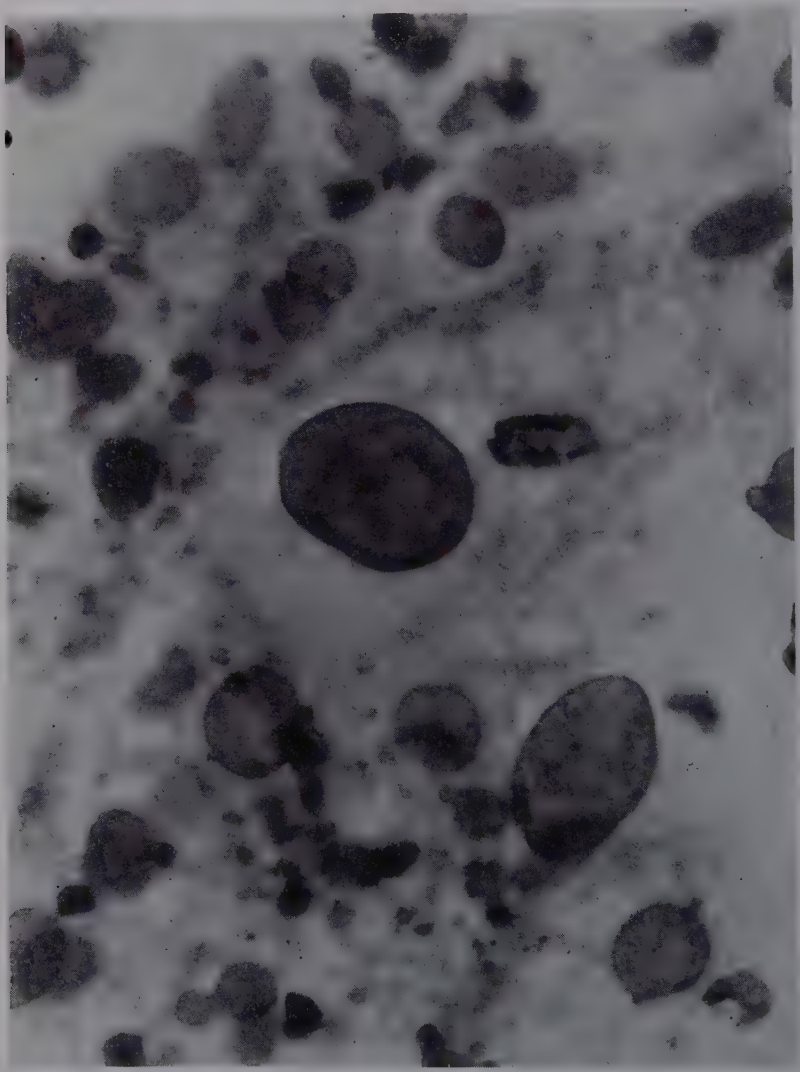


FIGURE 7. Cells appearing in the culture of a tumor B explant that was irradiated with 80,000 r. Note the great variation in size of the nuclei and the faintly distinguishable cytoplasm. Reproduced by permission of *Radiology*.

similar for both types of tumors. The rate of anaerobic lactic acid formation of the epithelial-type tumor C was more than twice that produced under aerobic conditions. The rate of anaerobic glycolysis of the spindle-cell tumor D was slightly higher than that of aerobic glycolysis. It was of particular interest to note that the oxidative phosphorylation of the isolated mitochon-

dria from epithelial-type tumor C cells was almost that of normal liver mitochondria (P:O ratio 2.14), while the oxidative phosphorylation of the mitochondria isolated from the spindle cell-type tumor D was very low. This

FIGURE 8. Cells in the outgrowth from an explant of tumor A that was irradiated with 100,000 r. Note the intact nuclei, nucleoli, and the homogeneous cytoplasm. This is in contrast to FIGURE 7. Reproduced by permission of *Radiology*.

observation indicated that the mitochondria of the spindle-cell tumor must be either fewer in number or of inferior quality as compared with those of the epithelial-type tumor.

Electronmicroscopic studies were carried out in order to obtain some information regarding this point. The electronmicrophotographs (FIGURES 13,

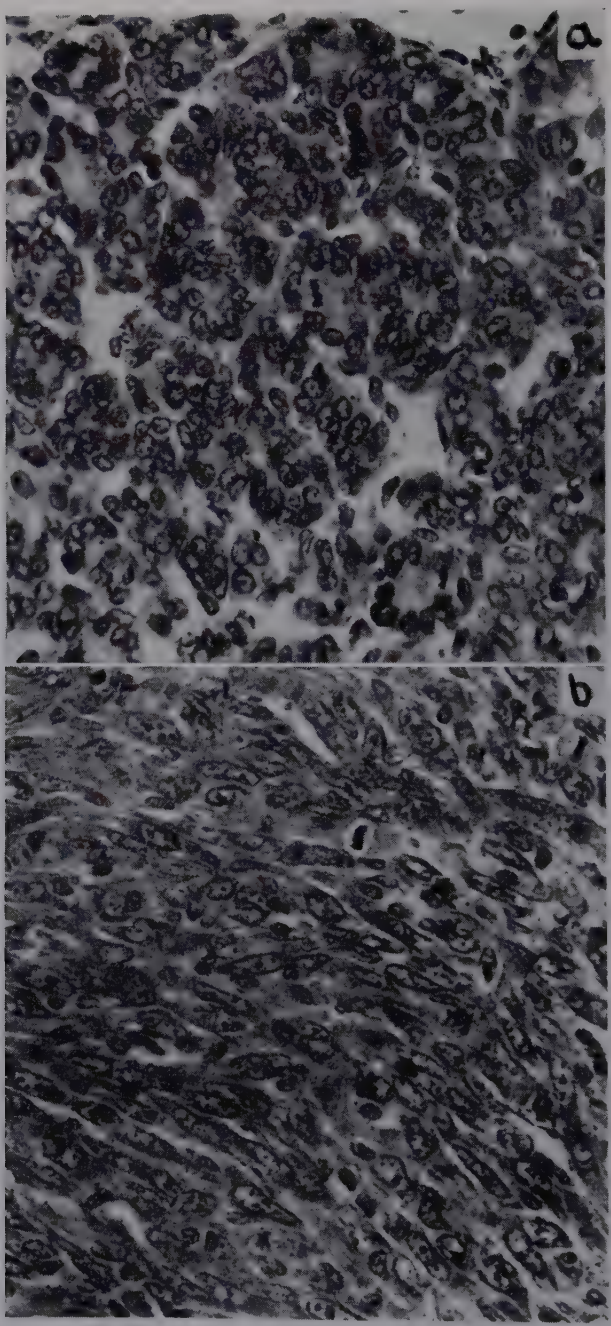


FIGURE 9. (*a*) A microscopic field of mouse mammary adenocarcinoma (tumor C); note the acini forms and nests of cells; the nuclei are rather small, mostly round, some oval, mitotic figures; and (*b*) microscopic field of mouse mammary tumor D; note the spindle-shaped cells, loosely distributed; the nuclei are rather large and elongated, mitotic figures.

FIGURE 10. Microscopic field of tumor D that was irradiated with 3000 r *in vivo*. Note the peculiar abnormal mitotic figures that appeared 72 hours after irradiation.

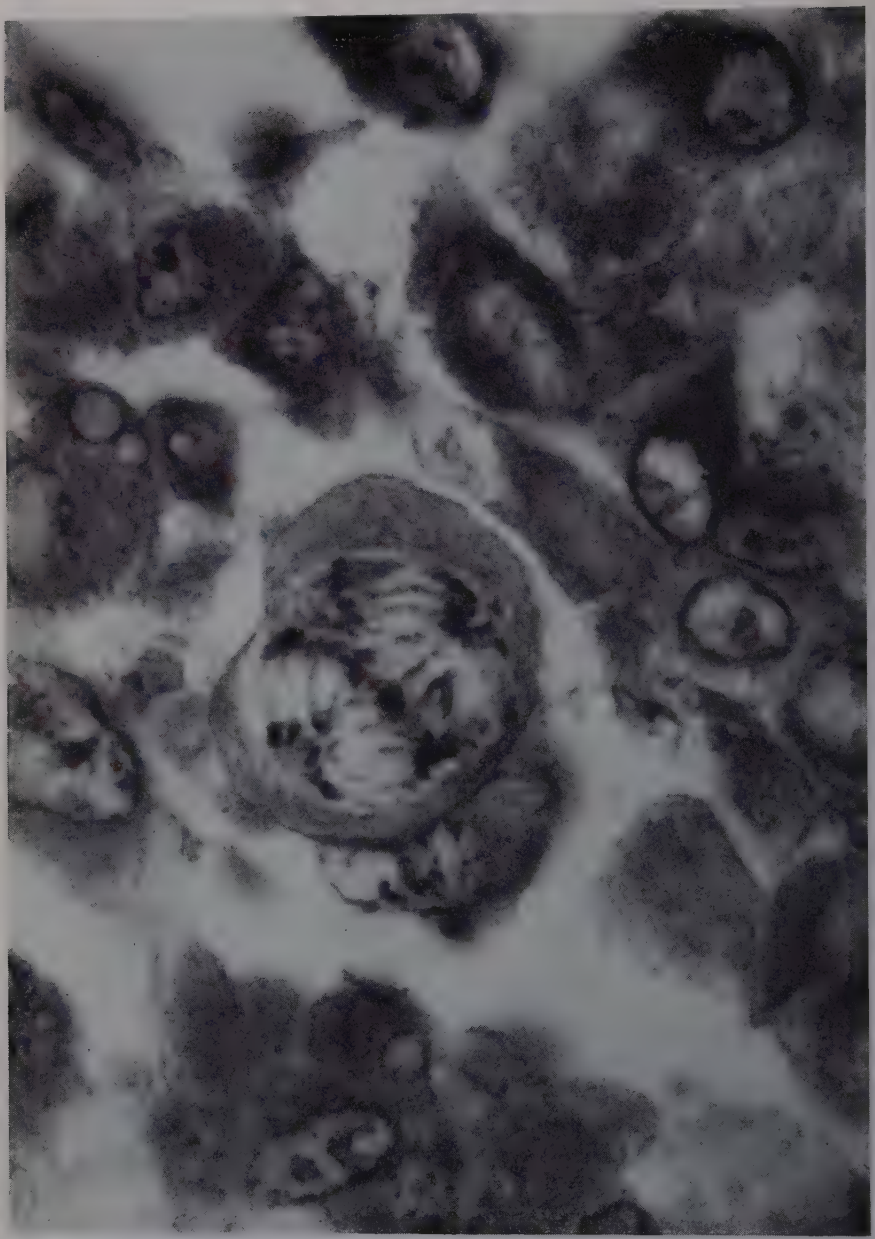


FIGURE 11. Microscopic field of tumor D that was irradiated with 3000 r *in vivo*. Note a tetrapolar mitosis, an unusual occurrence among mammalian cells. Reproduced by permission of the *International Journal of Radiation Biology*.

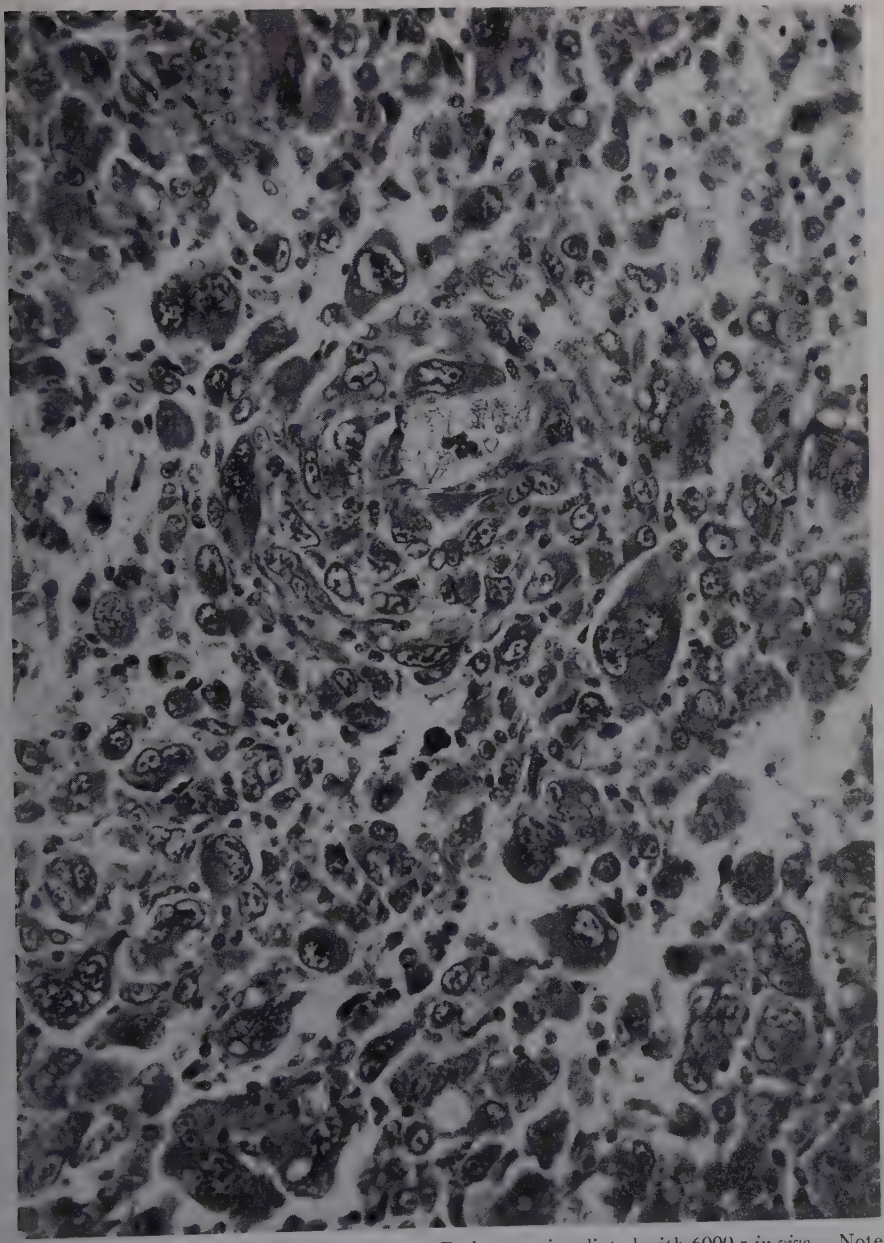


FIGURE 12. A microscopic field of tumor D that was irradiated with 6000 r *in vivo*. Note the various abnormal cells multinucleated with dense cytoplasm. These were seen in sections taken from the tumor 7 days after irradiation.

14, and 15) illustrate the difference in the quantity and quality of the mitochondria of these two types of tumors. It can readily be noted that the mitochondria are more numerous in the cells of the epithelial-type tumor C. Furthermore they are of a better quality, that is they are filled with christae mitochondrialis, which is a normal feature of healthy mitochrondria. In

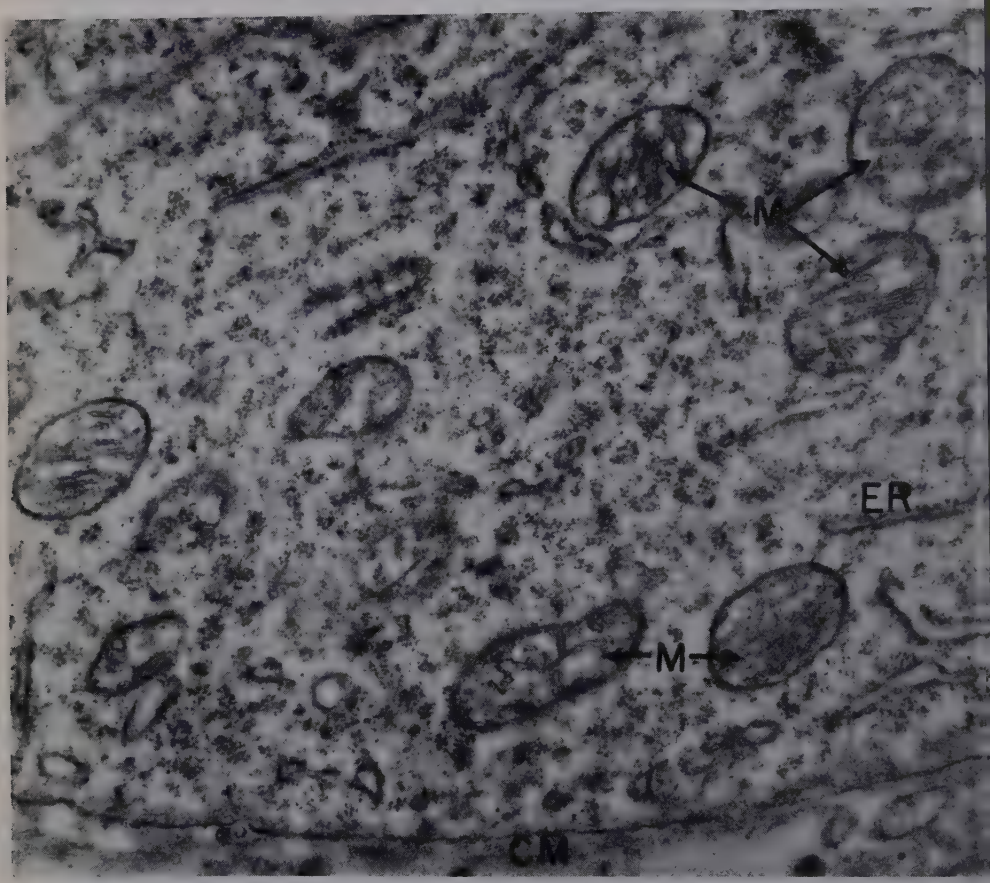


FIGURE 13. Electronmicrograph of an ultrathin section of tumor C. This illustration represents chiefly the cytoplasmic components. Note in particular the numerous mitochondria (*m*) with double membrane, filled with christae mitochondriales; the endoplasmic reticulum (*er*) in the form of vesicles and cisternae; numerous ribonucleoprotein particles (rnp) known as Palade particles; cellular membrane (*cm*); intercellular space; and a part of another cell.

contrast the mitochrondria of the spindle-cell tumor D (FIGURES 14 and 15) are fewer in number and contain less christae mitochondriales. Some of the mitochondria are small and empty, that is, free of christae. The scarcity and inferior quality of the mitochondria in the spindle cell-type tumor D may explain their low capacity to phosphorylate. The mitochondria are normally the main source of enzymatic reactions necessary for metabolic processes for production of energy for cellular function. It is therefore tempting to relate

FIGURE 14. Electronmicrograph of an ultrathin section of tumor D. Note in particular the irregular size and shape of the mitochondria (*m*) that contain few christae mitochondriales; the endoplasmic reticulum (*er*), and the rnp particles that are fewer in number as compared with FIGURE 13; part of a nucleus (*n*); and a double nuclear membrane (*nm*).

the difference in their quality and quantity to cellular radiosensitivity. This is an important point in my thesis in searching for an explanation for the observed difference in radiosensitivity among mammalian cells, normal or malignant.

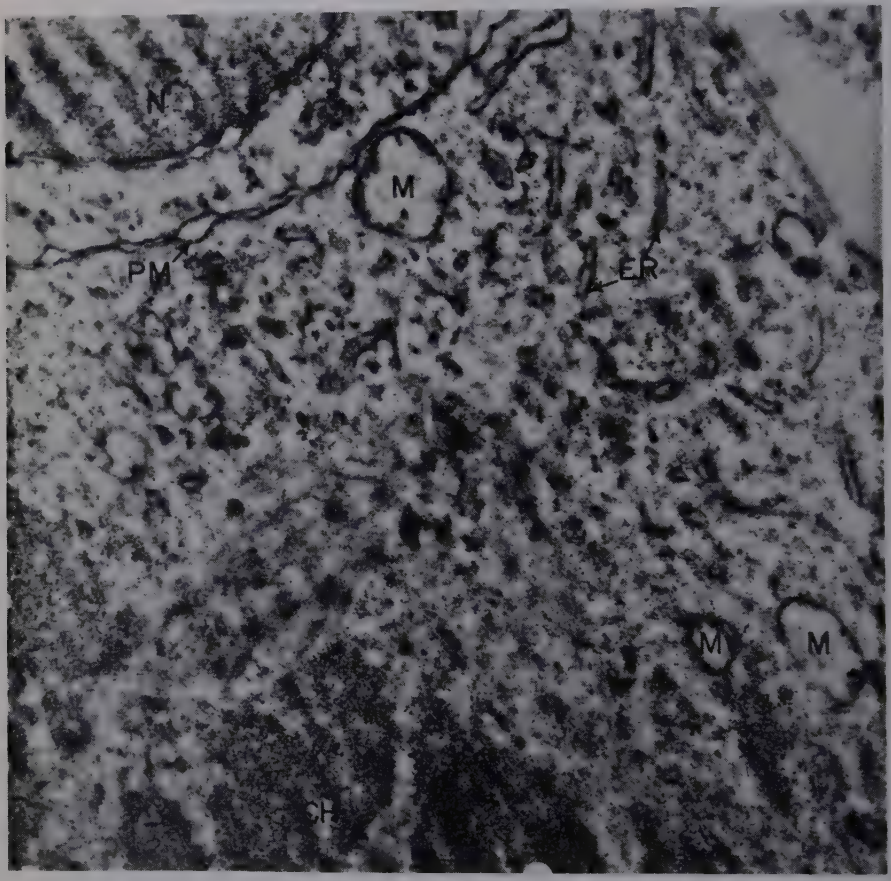


FIGURE 15. Electronmicrograph of an ultrathin section of tumor D is presented as another example. Note in particular the few mitochondria (*m*) that are empty, that is, free of christae mitochondriales; the endoplasmic reticulum (*er*); and the small rnp particles. Note also part of the nucleus (*n*); a nuclear membrane (*nm*); the cellular membrane (*cm*), and the plasma membrane (*PM*). Reproduced by permission of the *International Journal of Radiation Biology*.

Discussion

Doses ranging from 80,000 to 150,000 r/air were required for immediate suppression of growth *in vitro* of the tumor explants herein reported. The need for even larger doses to prevent growth *in vitro*, either of normal or malignant tissues, was previously noted by other investigators,[23-25] of whom only a few are cited. Pomerat *et al.*[26] recently reported that very high doses were

needed to achieve irreversible damage of monolayers of Henle's strain of cells *in vitro*. Approximately 140,000 r of gamma rays from cobalt-60 were required to destroy cells in the Rose chamber within 24 hours of incubation. Puck *et al.*,[11] using their single-cell plating technique, reported that the dose required to cause death of cells (the criterion being the failure to reproduce more than a few generations) is about 93 r for 37 per cent lethality of HeLa S3 cells and about 50 r for euploid human cells. It seems difficult to compare the results obtained by the use of the single-cell technique with those obtained by the use of complex tissues consisting of mixed-cell populations that are usually at various stages of physiological activity. This paper is mainly concerned with the response to irradiation of tissue aggregates rather than with the response of single cells grown in monolayer sheets.

It is important to mention at this point that recovery may take place in the cells following exposure to medium or sublethal doses of irradiation. This was noted by Lasnitzki[27] on chick fibroblasts using the classical hanging drop technique, and brilliantly demonstrated recently by Elkind *et al.*[28] on Chinese hamster cells by the use of the single-cell technique. The ability of cells to recover may explain the need for large radiation doses for destruction of whole populations in compact tissues.

A difference in radiosensitivity was observed between the tumor types as well as between normal embryonic tissues herein reported. It has already been noted by the earlier investigators that various cell types may differ in their response to irradiation. Bergonie and Tribandeau[29] formulated a concept on this basis that is expressed simply as follows: the sensitivity of cells to irradiation is in direct proportion to their reproductive ability and inversely proportional to their degree of differentiation.

Although this concept holds true for a number of tissue cells, there are exceptions to the rule. For example the lymphocytes, which are nondividing cells, proved to be radiosensitive; the polymorphonuclear leukocytes, which are differentiated cells, are also radiosensitive. There are other exceptions to this rule.[30] It follows that there may be factors other than the rate of growth and differentiation that influence radiosensitivity. Spear[31] in a recent review states: "There is a ten-thousandfold difference between the extreme sensitivity of different types of living cells when measured by the lethal effect. The reason for this is unknown." The question arises as to why cells differ in their response to irradiation.

As stated in the introduction the main objective of my investigations is to detect the inherent properties of cells that determine their radiosensitivity. The observations on the tumors reported here suggest that the intracellular composition and the physiological activity may play an important part in cellular radiosensitivity. Specifically the status of the mitochondria, which are most vital for metabolic activity of the cell, may play an important role in cellular radiosensitivity. As previously pointed out the mitochondria in the spindle-cell tumor D, which proved to be more radiosensitive, are fewer in number and inferior in quality. It may be expected that irradiation will still further impair the mitochondrial enzymatic activity necessary for synthesis of nutrients for cell life. This concept might explain the reason for the difference in radiosensitivity among various cell types, normal and malignant.

Thus far this study on a subcellular level has been exploratory, and future experiments will attempt to confirm and expand on these initial findings. Fundamental studies on a subcellular level may provide a definite explanation for the existent difference in radiosensitivity among various cell types, and may shed light on the mechanism(s) involved in the interaction of ionizing radiation with biological systems.

Summary

(1) A brief historical survey of radiation effects on tumor cells in tissue culture is provided.

(2) The radiosensitivity of various types of normal and neoplastic cells was studied.

(3) Cytological abnormalities in the irradiated cells, such as chromatin bridges, double anaphases in the same cell, tripolar and tetrapolar mitoses, multinucleated giant cells, nuclear and cytoplasmic vacuolization, and liquefaction of the culture medium were noted.

(4) Doses of X rays ranging from 80,000 r to 150,000 r were required for total inhibition of growth of the tissue explants in the cultures. Small doses permitted recovery.

(5) Evidence for a selective response to irradiation between various types of tissue cells, normal and malignant, is presented.

(6) Studies on a subcellular level by the aid of electronmicroscopic and microanalytic techniques revealed that the mitochondria of the more radiosensitive tumor are fewer in number and inferior in quality as compared to that of the more radioresistant tumor.

(7) The possible role these mitochondrial differences may play in cellular radiosensitivity is discussed.

Acknowledgments

I am indebted to the Physics Laboratory, Department of Hospitals, New York, N.Y., for physical measurements; to E. Hajjar for photographic work; to Gladys Cameron, who cooperated with me in part of this work; and particularly to George E. Palade of The Rockefeller Institute, New York, N.Y., for his invaluable cooperation in the electronmicroscopic work.

References

1. Wood, F. C. & F. Prime. 1914. The action of radium on growing cells. Proc. Soc. Exptl. Biol. Med. **11:** 140.
2. Price, J. C. & J. C. A. Mottram. 1914. A contribution to the study of *in vitro* plasma cultures of mouse carcinoma and rat sarcoma. Arch. Middlesex Hospital, London, England. **33:** 21.
3. Kimura, N. 1919. The effect of X-ray irradiation of living carcinoma and sarcoma cells in tissue culture *in vitro*. Am. J. Cancer. **4:** 95.
4. Love, W. H. 1931. Effect *in vitro* of homogeneous X irradiation on tissues. Arch. exptl. Zellforsch. **11:** 132.
5. Ludford, R. J. 1932. Cytological changes after irradiation of malignant growth. Tenth Sc. Report Imp. Cancer Res. Fund. London, England.
6. Strangeways, T. S. P. & H. E. Oakley. 1924. The immediate changes observed in tissue cells after exposure to soft X rays while growing *in vitro*. Proc. Roy. Soc. Ser. B. **95:** 373. London, England.
7. Perthes, G. 1904. Versuche uber den Einflus der Roentgenstrahlen auf die Zellteilung. Deut. med. Wochschr. **27:** 17.

8. Apolant, H. 1904. Über die Wirkung von Radiomstrahlen auf das Karzinom der Mause. Deut. med. Wochschr. **30:** 654.
9. Hertwig, G. 1911. Radiumbestrahlung unbefruchtener froscheier und ihre entwicklung nach befruchtung mit normalen samen. Arch. Microscop. Anat. **77:** 165.
10. Zircle, R. E. & W. Bloom. 1953. Irradiation of parts of individual cells. Science. **117:** 487.
11. Puck, T. T. & P. I. Marcus. 1956. Action of X rays on mammalian cells. J. Exptl. Med. **103:** 653.
12. Stroud, A. N. & A. M. Brues. 1954. Radiation effects in tissue culture. Texas Reports in Biol. & Med. **12:** 931.
13. Lasnitzki, I. 1958. Cancer cells in tissue culture. Cancer. **3:** 41.
14. Gey, G. O. 1941. Cytological observations on transplantable rat sarcomata produced by the innoculation of altered normal cells maintained in continuous cultures. Cancer Research. **1:** 737.
15. Earle, W. R. 1943–1944. Production of malignancy *in vitro*. J. Nat. Cancer Inst. **4:** 131.
16. Cameron, G. & R. Chambers. 1937. Neoplasm studies. III. Organization of cells of human tumors in tissue culture. Am. J. Cancer. **30:** 115.
17. Rozynek, M. 1938. Untersuchungen uber die fibrillenbildung des Jensen-Sarkoms *in vitro*. Virchow's Arch. **302:** 405.
18. Coman, D. R. 1942. Human neoplasms in tissue culture. Cancer Research. **2:** 618.
19. Leighton, J. 1954. The growth patterns of some transplantable tumors in sponge matrix tissue culture. J. Nat. Cancer Inst. **15:** 275.
20. Southam, C. M. 1954. Growth of human adenocarcinoma cells in tissue culture. Cancer. **7:** 394.
21. Maximow, A. Z. 1929. Über die Entwicklung argyrophiler und kollagener Fasern in Kulturen von erwachsenem Saugetiergewebe. (Nach des Verfassers Tode geschrieben und veröffentlicht von W. Bloom.) Z. Mikr. anat. Forschr. **17:** 625.
22. Goldfeder, A. 1938. Studies on the effects of radiation upon growth and respiration of various tissues *in vitro*. Radiology. **31:** 73.
Goldfeder, A. 1940. Further studies on the effects of irradiation on proliferation and metabolic processes of normal and malignant tissues. Radiology. **35:** 210.
23. Goldhaber, P., L. Doljanski & L. Halberstaedter. 1944. Comparative studies on the radiosensitivity of normal and malignant cells in culture. III. Further studies on the inhibitory effect of X rays on cell outgrowth. Cancer Research. **4:** 110.
24. Doljanski, L. & L. Halberstaedter. 1944. Comparative studies on radiosensitivity of normal and malignant cells *in vitro*. II. The delayed lethal effect. Cancer Research. **4:** 106.
25. Montgomery, O. B. & S. Warren. 1953. The ultraviolet microscopy of the living cell's response to lethal X radiation. Science. **117:** 589.
26. Pomerat, C. M. 1958. Cellular changes induced by radiation. Ann. N.Y. Acad. Sci. **71:** 1145.
27. Lasnitzki, I. 1943. The effects of X rays on cells cultivated *in vitro*. Part II. Recovery factor. Brit. J. Radio. **16:** 61.
28. Elkind, M. M. & H. Sutton. 1960. Repair of X-ray damage in surviving Chinese hamster cells. Radiation Research. **13:** 556.
29. Bergonie, J. & L. Tribandeau. 1906. Action des rayons x- sur les testicles. Arch. electric med. **14:** 779.
30. Bloom, W. 1947. Histological changes following radiation exposure. Radiology. **49:** 344.
31. Spear, F. G. 1958. Some biological aspects of experimental radiology. Intern. Rev. Cytol. Vol. VII.

SURVIVAL CURVES FOR TUMOR CELLS IRRADIATED *IN VIVO**

Harold B. Hewitt and Charles W. Wilson
Westminster Hospital Medical School, London, England

When we submitted the title of this paper, the question arose whether the paper was eligible for inclusion in a monograph the title of which specifies the use of cultures. In accepting this contribution it was evidently considered proper to include a paper dealing with the natural culture vessel in which animal tissue cells were ordained to be grown; that is, the whole animal. The procedure of isolating tissue cells from their natural environment and examining their reactions in tissue culture is of course one that is undertaken in order to provide a simpler and more controllable biological system for experimental studies. The ultimate objective must surely be to use the data obtained *in vitro* to elucidate the reaction of cells in their natural environment as part of the whole organism. Translation of the *in vitro* data back to the *in vivo* situation is certainly required where it is desired to explore problems associated with one of the principal applications of radiobiology: radiotherapy.

Since the pioneer work of Puck and Marcus (1956), who determined a survival curve for HeLa cells irradiated *in vitro*, these and numerous other workers have determined curves of a similar character for populations of many other different cell strains irradiated *in vitro*. Indeed, the response of these uniform populations of cells has proved to be very much more orderly than we were inclined to expect a few years ago. However, there were good reasons for supposing that the data obtained from *in vitro* studies might prove to be misleading guides to the course of events *in vivo*. For example: many of the cell strains that have been used are highly adapted to an artificial environment and could have an abnormal genetic constitution or metabolic pattern; indirect effects of radiation on cells, secondary to an effect on stromal tissues or on the host constitution, are necessarily excluded in a culture; and, finally, the *in vitro* criterion of cell survival—the ability to form a clone—may not be equivalent to the ability of a cell to proliferate *in vivo*.

Quantitative *in vivo* techniques for the determination of radiation survival curves naturally entail complications and uncertainties that do not trouble those who work with cultures, and we shall demonstrate some of these features in the experiments described here. Particular complications of these techniques which we have ourselves encountered are as follows: inability to define or control the oxygen status of cells *in vivo*; difficulties of releasing and distinguishing the specific cell type under examination from the heterogeneous population of cells to which they belong in the whole tissue; various manifestations of transplantation immunity may cause considerable confusion in interpretation of the results of the transplantation bioassays used in these techniques; and, finally, cells that have lost their reproductive integrity after irradiation may have a very significant influence on the assay of surviving

* The work described in this paper was supported by a grant from the British Empire Cancer Campaign, London, England.

intact cells with which they are mixed and from which they are morphologically indistinguishable.

Survival Curves for Mouse Leukemia Cells Irradiated in Vivo

The first *in vivo* radiation survival curve that we determined was for a lymphocytic strain of leukemia that arose spontaneously in a mouse of our CBA colony, and that was readily transplantable within the colony (Hewitt, 1958; Hewitt and Wilson, 1959). Mice with the advanced transplanted disease have dense infiltration of the liver sinusoids with leukemia cells, and almost pure suspensions of intact leukemia cells can be prepared from such livers by mincing them in a suitable medium. Transplantation bioassays of these cells were performed by injecting serial dilutions of counted suspensions into groups of mice and recording the incidence of leukemia in the injected groups; from these data were calculated the mean number of morphologically intact cells required to transplant leukemia to one half of a group of injected mice. This value we call the TD50; and the mean TD50 obtained for a series of assays of cells from unirradiated mice was only 2 cells. When we exposed leukemic mice to whole-body Co^{60} gamma radiation and bioassayed the liver leukemia cells within one-half hour of their exposure, the TD50 values obtained were greater than 2, and were a function of the dose of radiation received by the donor mouse. The survival-rate values were calculated from the TD50 values before and after irradiation, and FIGURE 1 shows the survival curve that we obtained. It is of the same character as the curves that have been produced by others for tissue cells irradiated *in vitro*. No alteration of the radiosensitivity of the cells ensued when the mice were given 95 per cent oxygen instead of air to breathe during irradiation. It is reassuring to note also that the single survival rate obtained by L. H. Gray and ourselves for these cells irradiated under well-oxygenated conditions *in vitro* and assayed *in vivo*, did not depart from our *in vivo* curve. The slope of the linear part of the curve is not significantly different from that of the common survival curve obtained by Morkovin and Feldman (1960) for 6 different strains of human-tissue cells (including the HeLa strain) irradiated under dosimetrically workable and well-oxygenated conditions *in vitro*.

Using similar methods we determined a survival curve (FIGURE 2) for the same strain of leukemia cells irradiated in the livers of leukemic mice that were killed a few minutes before irradiation, under which conditions we can assume that the cells would be anoxic. The single point obtained by Gray and ourselves for the cells irradiated under anoxic conditions *in vitro* does not depart significantly from our curve for irradiation in dead mice. A comparison of our two survival curves, for irradiation respectively in living and dead mice, shows that, for production of a given survival rate under anoxic conditions, the dose of radiation required is greater than that required for an equal effect *in vivo*, by a factor 2·3.

The particular tumor host system used for the studies we have described was ideal, in the sense that it enabled us to avoid all the complications of this approach that we have referred to: the TD50 for the unirradiated cells was very low; the leukemia cells were easily released from the tissue they were

infiltrating and could be reliably identified; there was no evidence of transplantation immunity; and radiation-killed cells did not influence the proliferative capacity of viable cells injected in mixture with them. Because these conditions were ideal, these two curves appear to provide specially reliable fundamental data, and we shall refer to them as our "primary" curves and shall use them in subsequent figures as a background for data obtained using different tumors.

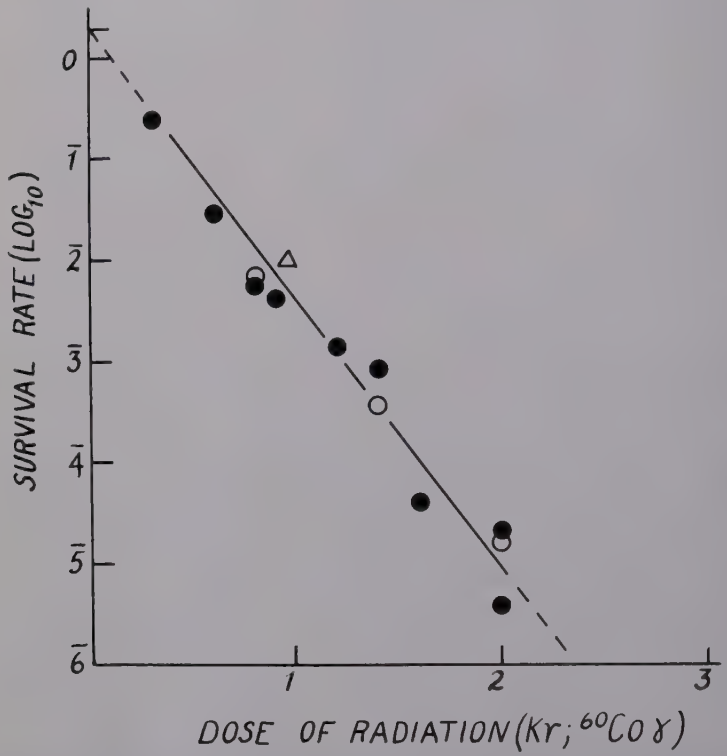


FIGURE 1. Survival curve for CBA leukemia cells irradiated under well-oxygenated conditions. Key: ●, cells irradiated *in vivo* in mice breathing air; ○, cells irradiated *in vivo* in mice breathing 95 per cent oxygen; and △, cells irradiated *in vitro* in a well-oxygenated medium.

We should mention here that we have used our *in vivo* primary curve successfully to predict "cure" rates among groups of mice that, at the time of exposure to 1600 r Co^{60} gamma radiation, were bearing known mean numbers of viable leukemia cells. The irradiated mice were protected against the lethal effects of the radiation by their intravenous injection with isologous bone marrow (Hewitt and Wilson, 1960).

A rather surprising conclusion that we must make from our two primary curves is that the leukemia cells infiltrating the livers of heavily leukemic mice were responding as a predominantly well-oxygenated population of cells,

despite their rapid rate of proliferation and metabolism and the fact that they were densely packed in the liver sinusoids.

Experiments with Circulating Leukemia Cells

It naturally was of interest to us to know whether leukemia cells situated in other organs of the leukemic mouse would respond to radiation in accord-

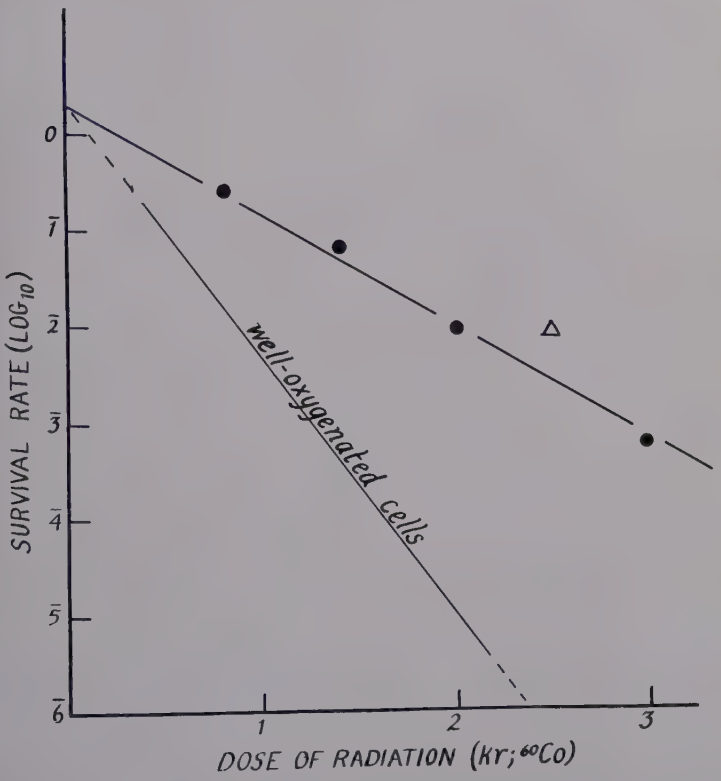


FIGURE 2. Key: ●, survival curve for CBA leukemia cells irradiated in recently-killed mice; and △, under anoxic conditions *in vitro* (the lower curve is superimposed from FIGURE 1).

ance with our *in vivo* primary curve. In the later stages of the transplanted disease, leukemia cells appear in moderate numbers in the circulating blood, in which their density is easily measured. When we determined the TD50 of circulating leukemia cells from unirradiated mice we again obtained values close to 2 cells. Survival rates obtained for the circulating leukemia cells after irradiation, and which for technical reasons had to be confined to the lower dose range, were found to give points whose distribution accorded with our anoxic curve rather than our well-oxygenated curve (FIGURE 3). This was a most unexpected finding because Gray (1957) has shown that relative radioresistance of mouse tumor cells due to hypoxemia is insignificant until the

oxygen tension in the environment of the cells falls below about 20 mm. Hg, which is distinctly lower than the oxygen tension in the systemic arteries or veins of mammals. In an attempt to explain this anomaly we entertained the hypothesis that the leukemia cells that we removed from the circulation

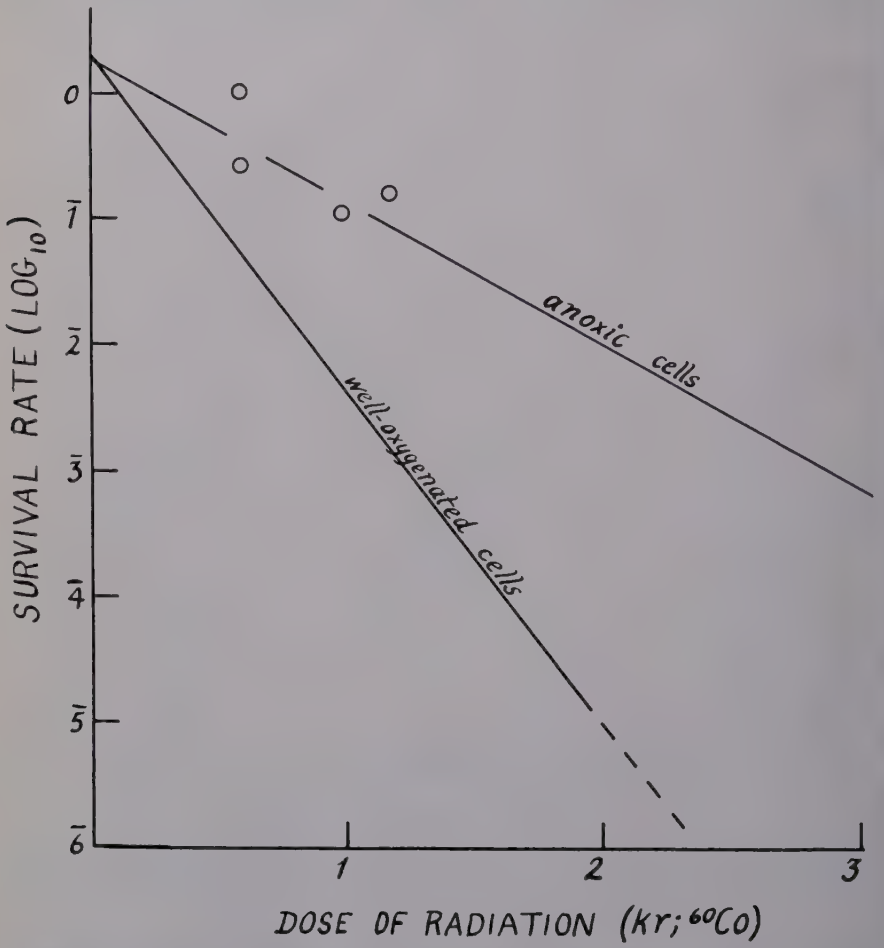


FIGURE 3. Survival rates among circulating leukemia cells of leukemic mice (the drawn curves are superimposed from FIGURES 1 and 2).

about 20 min. after irradiation had not been in the circulation during irradiation but were in some anoxic sites outside the circulation. However, since the density of cells in the blood remained fairly constant over a period of hours, our hypothesis requires us to postulate that leukemia cells were being turned over fairly rapidly in the circulation, being constantly fed into it from as yet unidentified anoxic sites and being removed elsewhere. We obtained strong evidence in support of our hypothesis by showing that radiochromium-

labeled leukemia cells left the circulation very rapidly after their intravenous injection, whereas similarly labeled erythrocytes were very largely retained in the circulation (TABLE 1).

These findings for the circulating leukemia cells have interesting implications: (1) it is apparent that radiobiological studies of this kind can give indirect information about the oxygen tension in various anatomic sites *in vivo*, and may serve to disclose unexpected physiological or pathological mechanisms; and (2) it is important to note that tumor cells may sometimes be under hypoxic conditions *in vivo* even where, as in the case of this leukemia, we have no large areas of massive necrosis such as are commonly seen in many large solid tumors.

TABLE 1

ESTIMATED PERCENTAGES OF TOTAL INJECTED RADIOACTIVITY REMAINING IN CIRCULATION AFTER INTRAVENOUS INJECTION OF Cr^{51}-LABELED CELLS INTO MICE

Time after injection (min.)	Per cent activity remaining	
	Cr^{51} erythrocytes	Cr^{51} leukemia cells
4	97.8	—
10	—	0.6
30	100	0.3, 5.0, 2.5
60	100	—
70	—	0.3
88	97.4	—
116	88	—

Survival Rate Data for Other Strains of Leukemia

We have used identical methods to measure the survival rates after irradiation *in vivo* of several other strains of mouse leukemia. Limitation of our supplies of pure line mice have prevented our determining complete survival curves for these other strains, but the few points we have obtained to date (FIGURE 4) indicate that the differences of mean lethal dose of radiation for different strains may not be great. Three of these additional strains were radiation-induced; one was a mutant strain derived from our original leukemia, which gave us the primary curves and was distinguished from the parent strain by its exhibiting a significantly lower growth rate and a distinctly greater mean cell diameter. One of the points shown in FIGURE 4 was obtained for a subline of our primary leukemia that had been repeatedly irradiated *in vivo* and regenerated from the survivors after each exposure. Exposure to repeated doses totaling about 20,000 r Co^{60} gamma radiation evidently failed to give a significant increase of radioresistance.

A Survival Curve for a Mouse Sarcoma Irradiated in Vivo

Finally we propose to describe a series of experiments done to determine a survival curve for the cells of a solid round-celled sarcoma irradiated *in vivo*. The tumors studied were isologous subcutaneous transplants from standard-

sized inocula, and were 0.5 to 1.0 cm. in diameter at the time of irradiation. Single-cell suspensions of morphologically intact sarcoma cells were prepared by enzymatic digestion of whole minced tumors. The density of the cells was determined by direct counting under phase-contrast microscopy, and the cells were bioassayed by injecting serial dilutions of the suspensions subcutaneously into groups of mice. The number of morphologically intact sarcoma cells required for 50 per cent of takes (the TD50) averaged 85 cells in a

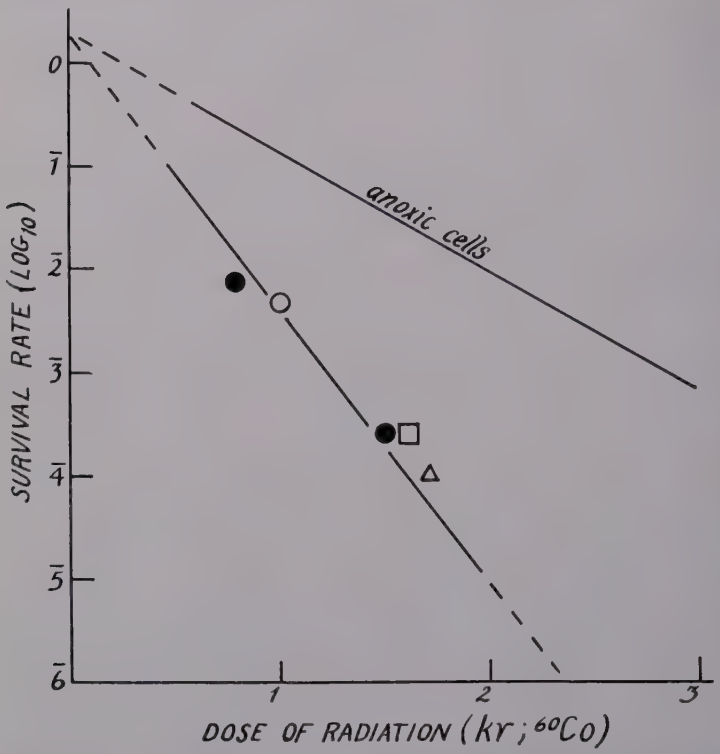


FIGURE 4. Survival rates for further leukemia strains irradiated *in vivo*. Key: ● ○ △ three radiation induced leukemia strains; and □, subline of leukemia that had been repeatedly irradiated and regenerated (the drawn curves are superimposed from FIGURES 1 and 2).

series of assays of cells from unirradiated tumors. Assays of cells from tumors recently irradiated *in vivo* gave TD50 values that were greater than 85, and were a function of the dose of radiation to which they had been exposed. The survival rates for irradiated cells were calculated from the expression: 85/TD50 for irradiated cells. When the logarithms of these values were plotted against the relevant doses of radiation, we obtained a survival curve (FIGURE 5, encircled points) that was more or less parallel to our anoxic curve for the primary leukemia but that lay significantly above it. We concluded tentatively that we were dealing with cells that were predominantly anoxic *in vivo* and whose radiosensitivity was characterized by a mean lethal dose of

radiation similar to that of our primary leukemia. The very high extrapolation number given by this curve was believed to be spurious and to have resulted from some complicating influence affecting the validity of the TD50 values. Such proved to be the case. When unirradiated sarcoma cells were assayed after mixing them with a preponderance of nonviable heavily irradiated cells, the TD50 value fell from 85 to only 5 cells. Now since the cell suspensions we assayed from irradiated tumors would in fact consist of surviv-

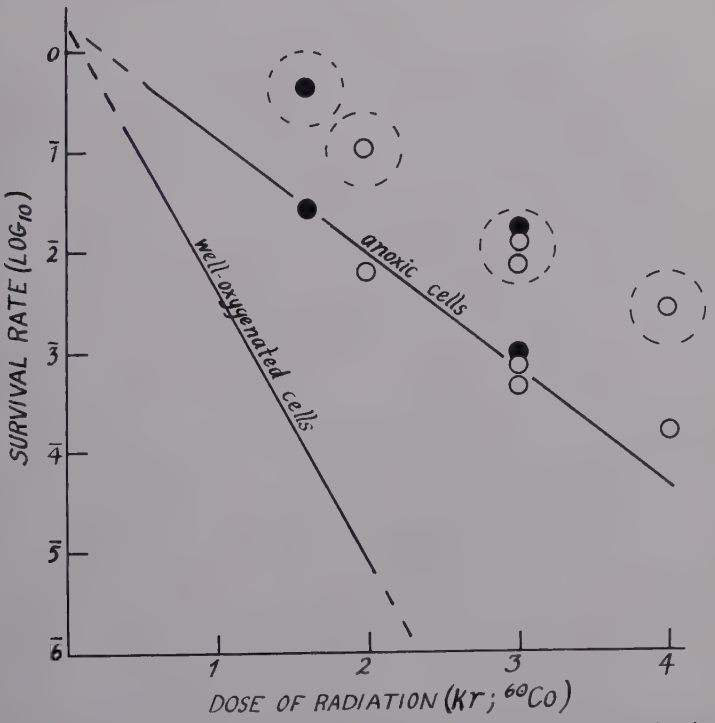


FIGURE 5. Survival rates for CBA sarcoma cells irradiated. Key: ○, *in vivo* in mice breathing air; and ●, in recently killed mice (the encircled points represent plots based on inappropriate calculations of the survival rate, as described in the text; the drawn curves are superimposed from FIGURES 1 and 2).

ing reproductively intact cells mixed with a preponderance of radiation-killed cells, it was clearly more correct to calculate our survival rate values as 5/TD50 for irradiated cells instead of 85/TD50 for irradiated cells. When this was done and the logarithms of the newly calculated survival values were plotted against dose of radiation (FIGURE 5), the points did not depart significantly from our primary anoxic curve for the leukemia. It remained for us to show that the tumor cells *in vivo* were in fact responding as an anoxic population. We did this by obtaining 2 further points for the cells irradiated in tumors carried on recently killed mice, under which conditions we can assume the cells were anoxic. These points (FIGURE 5) were in line with our *in*

vivo points for this tumor, and we were obliged to assume from our studies that the sarcoma cells were mostly anoxic even when growing on living unanaesthetized mice. We should add that the relative accuracy of our data does not permit us to state that 100 per cent of the cells were anoxic *in vivo*; our results are compatible with slightly less than one half of the cells being anoxic. Nevertheless this finding has very important implications for the radiotherapy of clinical tumors and is of considerable interest in relation to the metabolism of tumors.

Conclusions

We feel that the results of these *in vivo* studies are reassuring, in the sense in which they show that the curves obtained by irradiation of cells *in vitro* are in no way misleading. *In vitro* methods have the advantage of permitting much more accurate definition of basic parameters for the irradiation of cell populations. In this connection we should point out that a single mouse in our leukemia experiments gives only a quantal response and contributes to the numerical data no more than a single clone in culture.

Reviewing our rather incomplete data up to the present time, we have been impressed by the similarity of the responses of the four or five tumors we have studied, all of which may prove to conform to a single survival curve. The survival curves for numerous human-tissue cell strains irradiated *in vitro* that have been described by Puck *et al.* (1957) are also characterized by their similarities, although these workers did describe a small group difference between fibroblastic and epithelial cell types. Morkovin and Feldman (1960) showed that 6 human cell strains, including the malignant strain HeLa, conformed to a single survival curve when irradiated *in vitro* under well-oxygenated conditions. Moreover this curve was not significantly different from our *in vivo* primary curve for the mouse leukemia. Certainly no differences of survival curve slope have been described for different cell strains of man or mouse, irradiated under similar conditions, which approach the difference, for any one strain, which is attributable to the oxygen factor. The oxygen status of tumors emerges as the paramount influence affecting the dose of radiation required to eradicate the growth potential of a tumor *in vivo*. *In vivo* systems are therefore indispensable for exploring outstanding problems in radiotherapy.

Acknowledgments

We are indebted to R. J. V. Pulvertaft for valuable advice and encouragement; to J. J. Gough and J. E. Burns for assistance with the irradiation procedures; and to E. Blake and D. Levy for assistance with the biological work.

References

Gray, L. H. 1957. Oxygenation in radiotherapy. (i) Radiobiological considerations. Brit. J. Radiol. **30:** 403.

Hewitt, H. B. 1958. Studies of the dissemination and quantitative transplantation of a lymphocytic leukemia of CBA mice. Brit. J. Cancer. **12:** 378.

Hewitt, H. B. & C. W. Wilson. 1959. A survival curve for mammalian leukemia cells irradiated *in vivo* (implications for the treatment of mouse leukemia by whole-body irradiation). Brit. J. Cancer. **13:** 69.

Hewitt, H. B. & C. W. Wilson. 1960. Further studies relating to the implications of radiation survival curve data for treatment of CBA mouse leukemia by whole-body irradiation. Brit. J. Cancer. **14:** 186.

Morkovin, D. & A. Feldman. 1960. End point of one of the actions of radiations on living tissue important in radiation therapy and in acute radiation syndrome. Brit. J. Radiol. **33:** 197.

Puck, T. T. & P. I. Marcus. 1956. Action of X rays on mammalian cells. J. Exptl. Med. **103:** 653.

Puck, T. T., D. Morkovin, P. I. Marcus & S. J. Cieciura. 1957. Action of X rays on mammalian cells. II. Survival curves of cells from normal human tissues. J. Exptl. Med. **106:** 485.

RADIOBIOLOGIC OBSERVATIONS ON HUMAN HEMIC CELLS *IN VIVO* AND *IN VITRO**

Edwin E. Osgood

Division of Experimental Medicine, University of Oregon Medical School, Portland, Ore.

This paper summarizes 30 years of study of the action of ionizing radiation on human hemic cells *in vivo* and *in vitro*. Only the conclusions and references to our own work can be given here. These references cite the earlier relevant studies of others. Most of these conclusions have been reached independently by many others on the basis of different evidence either before[1] or after these studies.

The development of the concept[2-6] of the alpha cell and the n cell has clarified my own understanding of previously reported observations. The alpha cell of any series is capable of division, and remains immature to divide again. It is the only cell that can start a culture or a transplant. It is potentially immortal. The alpha cell may undergo 2 types of division: alpha-2 alpha that eventually doubles the population, or alpha-n that maintains a constant ratio of alpha to n cells. The n cells mature, function, and die in a time that is short relative to the life span of the species; they may or may not undergo n-2n division,[6] but these are all lost to genetic continuity. Only the alpha cell maintains somatic genetic continuity.

All of our studies both culture and clinical have been done with a dosage of ionizing radiation that is within the range of 10 r to 400 r, or its equivalent in other modalities, unless specifically stated otherwise. The conclusions may not apply to higher levels of radiation. We chose this range because it is the range of clinically effective single-dose therapy.

The action of such irradiation is instantaneous and direct on the intermitotic alpha cell to inhibit alpha-n division.

The culture evidence that the action is direct[7] is that interchange of media between an irradiated and unirradiated culture fails to show any effect on the nonirradiated cells with medium from an irradiated culture. Clinical evidence is that shielded tissues are protected and that X ray or P^{32} have equivalent effects,[8-14] although the radiation is given at very different rates. This does not of course rule out an effect mediated by active radicals with a life span of only a few seconds and a range of effect of only a few microns.

That the action is on the intermitotic alpha cell is shown in culture[15,16] by that fact that the quantitative effects of colchicine alone, colchicine plus X ray, and X ray alone, at doses of each agent that inhibit essentially all cell division when compared to a control handled identically, but with no colchicine or X ray, are identical. No decrease occurs in cells incapable of division[17] until enough time has elapsed for cells to develop to this stage. The sequence

* The work described in this paper was supported in part by Research Grant CY-3374 from the National Cancer Institute, Public Health Service, Bethesda, Md. Contract AT(45-1)-581 from the United States Atomic Energy Commission, Washington, D.C. and the Medical Research Foundation of Oregon, Portland, Ore.; and Postdoctoral Foreign Fellowship Grant FFM-4 from the National Institute of Mental Health, Public Health Service.

of drop is the sequence of the stage of development of the n cell, just as one would note, if one stopped children from entering the first grade, a drop in the population in the first grade long before there was any change of population in the eighth grade. Clinically[10,12] this is shown by the time lag before the peripheral drop in leukocyte counts occurs that is related to the life span of the corresponding cell type, and by the fact that the effects[18] are far too great to be accounted for by action only on cells that were in process of division at the time of a very brief irradiation.

The fact that the action is to inhibit alpha-n divisions is shown by the same experiment[15,16] in that the control cultures showed 1.5 per cent mitoses, the X ray alone no mitoses, the colchicine alone 57 per cent of all cells capable of division arrested in metaphase, and the colchicine plus X ray no cells arrested in metaphase. Obviously prevention of alpha-n division in the intermitotic stage or arrest in metaphase would give the same quantitative effect. Furthermore all 3 types of cultures showed the same sequence[17] and slope of drop relative to the life span of the n cell of the corresponding series. This is an arithmetic drop if the patient was in a steady state and X ray was used as the therapy. Clinically the drop after the same dose of irradiation is much more rapid in acute leukemia, where the cell life span is greatly shortened, than in chronic leukemias[19-24] of the same type where there is only a moderate shortening of the life span of the n cell, or in polycythemia vera[12,25] where the erythrocytes have a normal life span.

Furthermore the action is not apparent until cell division begins, as shown by the fact that cultures after irradiation kept at room or refrigerator temperature show identical counts within the limits of counting error until placed in the incubator.[26] Only after incubation is the difference in cell counts apparent. This is shown clinically by the fact that cells known not to divide are extremely radiation-resistant and that the killing dose for n cells is many orders of magnitude larger than the dose that produces a significant drop in time in the n cells in all series except possibly the lymphocytic series, where there is some evidence that small doses may kill cells.

Studies, both culture and clinical, have shown that for the hemic cells of man the order of radiation sensitivity[10] from the most sensitive to the least sensitive is as follows: leukemic series: lymphocytic > granulocytic > monocytic > plasmocytic > polycythemic erythrocytic >; normal: lymphocytic > granulocytic > thrombocytic > erythrocytic > monocytic > plasmocytic.

Further evidence that the action is only on the alpha cells is that the effect of a single dose of radiation persists far longer than the life span of the n cell of that series either in culture or clinically.[12,18] In irradiated cultures, after a single dose, counts drop and then become stable for a considerable time before mitoses begin again. For a period of time, only the most immature cells of the series, those containing nucleoli, are present. The clinical evidence is that one may need to treat patients with polycythemia vera[10,12] only at intervals of 6 mo. to 5 years, even though the life span of the n cell of this series is only 120 days; and in chronic granulocytic leukemias[18] the treatment may be given at intervals of as long as 12 weeks, when the life span of the leukemic n cell of this series is of the order of 5 days.

Cultures are well adapted for studies on the relative biological effectiveness of different modalities of ionizing radiation. We determined in culture[10,15,16,26] that 1 million-v X rays and 200 kv X rays had identical effects per r reaching the cells, that 1 N of neutrons equalled 4 r, and that 1 μc. of P^{32}/ml. in cells exposed for 24 hours was equivalent in effect to 35 r; and we have unpublished clinical data to indicate that 1 mc. of P^{32} administered I.V. in adults of either sex in either chronic lymphocytic or chronic granulocytic leukemia is equivalent to 15 r of total body irradiation. The difference between the culture equivalent and the clinical equivalent is due to several factors: the excretion of P^{32} by the patient,[12,19-22] the fact that it equilibrates with only about 56 gm. of the 700 gm. of P^{31} in the body, and the fact that, in the body, incorporation in the cell continues after 24 hours.

One of the most interesting observations both in culture and clinically is the extraordinary difference in sensitivity of populations of cells of a single series within 1 individual at 1 moment of time,[10,15,26] in the same individual at different times, and between individuals. In culture we could obtain quantitatively measurable effects on the lymphocytic series with 10 r, but could not eliminate the last cell of the lymphocytic series with 2000 r. This undoubtedly accounts for the fact that no dose of total body irradiation thus far given has been successful in eliminating leukemia. That individuals may suddenly change in their dose requirement to either a smaller or larger dose is well documented in our series of more than 500 chronic leukemias (Osgood and Seaman, Figure 1B;[9] Osgood, Figure 2[18]) treated with regularly spaced titrated ionizing radiation. The dose may vary between individuals with the same type of leukemia[18] and with the same initial cell count by a factor of more than 10 based on many years of follow-up. We have also shown in culture that while the changes in the peripheral blood may appear similar, the action of nitrogen mustard[17] and urethane[27] is not the same as that of radiation. Radiation inhibits division in the alpha cell, and the effect increases with the logarithm of the dose over a very wide range. Nitrogen mustard kills all cells of the granulocytic series except the segmented neutrophil as shown by a semilogarithmic, not an arithmetic, drop beginning in 3 hours, not much later, as with radiation. Furthermore nitrogen mustard tends to have an all-or-none effect with no measurable effect in culture up to a certain concentration and an almost maximal effect by doubling this concentration. Cell death occurs largely by pyknosis and lysis of the cells. Urethane leads to the progressive and much slower appearance of very abnormal mitoses with marked karyorrhexis of cells and many cells with double nuclei, and the effects occur much more gradually than with mustard and persist for a long time after the drug is stopped.

The heretofore unpublished culture observations presented in more detail are three: (1) we have confirmed Lajtha's observation[28] that DNA synthesis occurs in mid-interphase; (2) we have confirmed the finding that irradiation in the clinical range does not inhibit DNA synthesis; and (3) we have much evidence that any interpretation of the biologic effect of ionizing radiation must take into consideration the ecology[2,29-32] of the particular cell type being studied. FIGURE 1 is a modified diagram of Lajtha's concept of the sequence

of events in the intermitotic cycle of the alpha cell with our concept of the n cell added to the diagram. Note that immediately after 1 cell division of an alpha cell there is a period of time called G_1 by Lajtha during which the DNA of the cell is at the diploid level. Then there is a period during which DNA synthesis occurs until the amount of DNA has doubled; Lajtha calls this period the S period, or synthetic period. Following this, there is a second interval that Lajtha calls G_2, during which no DNA synthesis occurs. Only during the S period is tritium-labeled thymidine incorporated in the alpha cell. Then division occurs to produce usually 1 alpha cell and 1 n cell reducing the total label to one half of that present just before division and reducing the total DNA of the cell correspondingly. Just before the division the cell is double the normal diploid volume and contains double the normal amount of DNA of the mature n cell, as shown in FIGURE 2. If this is an alpha-n division and there are no n-n divisions,[6] the n cell retains the label for its life

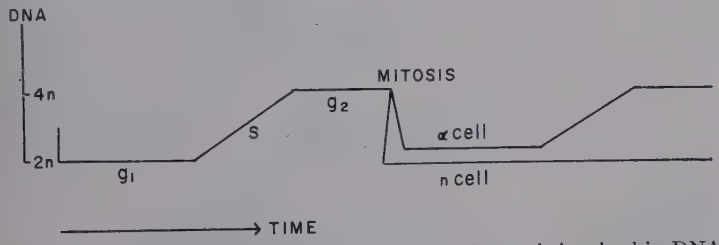


FIGURE 1. Diagrammatic representation of the time intervals involved in DNA labeling adapted from Lajtha *et al.*[28] During G_1 and G_2 periods no DNA synthesis is carried on, and no label enters the DNA. During the synthetic period S the amount of DNA doubles from the 2N to the 4N amount and the label is incorporated at this time. The mitosis following the G_2 period results in 1 alpha cell destined to divide again and 1 n cell destined to differentiate and die. These cells each carry one half the label of the 4N state preceding division. The n cell will never get more; the alpha cell, with successive divisions, will reach 50 per cent, 75 per cent, and 87.5 per cent, for example, of the maximum label possible for the 4N or 2N state.

span but never accumulates any further label (see Osgood, Figures 4 and 8[5]), while the alpha cell repeats alpha-n divisions for a variable number of times, and may also undergo alpha-2-alpha divisions that may be recognized since the total population of cells will double at this time. After 1 life span of the n cells, the cell count remains constant if only alpha-n divisions are occurring. The alpha cell, whether the divisions are alpha-n or alpha-2-alpha, if the concentration and specific activity are held constant, reaches 50 per cent of its final label after the first division, 75 per cent after the second division, 87.5 per cent after the third division, continuing in this way to essentially 100 per cent label by 6 divisions. Labeling during the S period is extremely rapid so that in periods of time as short as 10 min. all alpha cells that were in the S period are labeled. From that time on the rate of labeling corresponds to the rate at which alpha cells enter the S period. Until after the lapse of the first G_2 period no labeled mitoses will be found. Until the lapse of the longest G_2 period, some unlabeled mitoses will be found (see FIGURE 2). This shows an unlabeled metaphase surrounded by 3 heavily labeled cells 13 hours after tritiated thymidine was added to a rapidly growing culture of human leukemic

granulocytic cells. Disintegrated dead cells will not begin to show a label until after the shortest life span of the n cell has been reached, and will not all be labeled until after the longest life span of the n cell has been reached, after which time disintegrated fragments of labeled chromatin may be found phagocytosed in the cytoplasm of cells. My associate, D. A. Rigas, has developed equations for such isotope incorporation.[33,34]

The fact that irradiation does not inhibit DNA synthesis is demonstrated by the observation that cells continue to increase in size (FIGURE 3*a* and *b*) and to become labeled when increase in number has ceased. That is of course the mechanism by which Puck[35] developed his feeder layer of cells,

FIGURE 2. Autoradiograph of a cover slip with cells grown for 13 hours in medium containing tritiated thymidine 1 μc./ml. Unlabeled mitotic figure and heavily labeled nuclei. Culture strain[30] $J111C1_{1552}$.

and that is why it is essential in using tritiated thymidine to have a low enough total activity and specific activity to reduce the final concentration attained in the alpha cell below the threshold for inhibition of cell division. These observations explain why tritiated thymidine given as a single dose in man labels only the alpha cells that happen to be in the synthetic period at the time it was given, since in man, unlike the cultures, the tritiated thymidine is quickly catabolized,[36] presumably in the liver, to tritiated water and other compounds that do not give DNA labeling.

The crucial importance of considering the ecology of the cell within the culture and within the body is demonstrated by FIGURES 4*a*, *b*, and *c* and TABLE 1. In this experiment tritiated thymidine was added to a gradient culture of rapidly growing cells of the plasmocytic series ($J181_{280}J111_{1396}$). The gradient

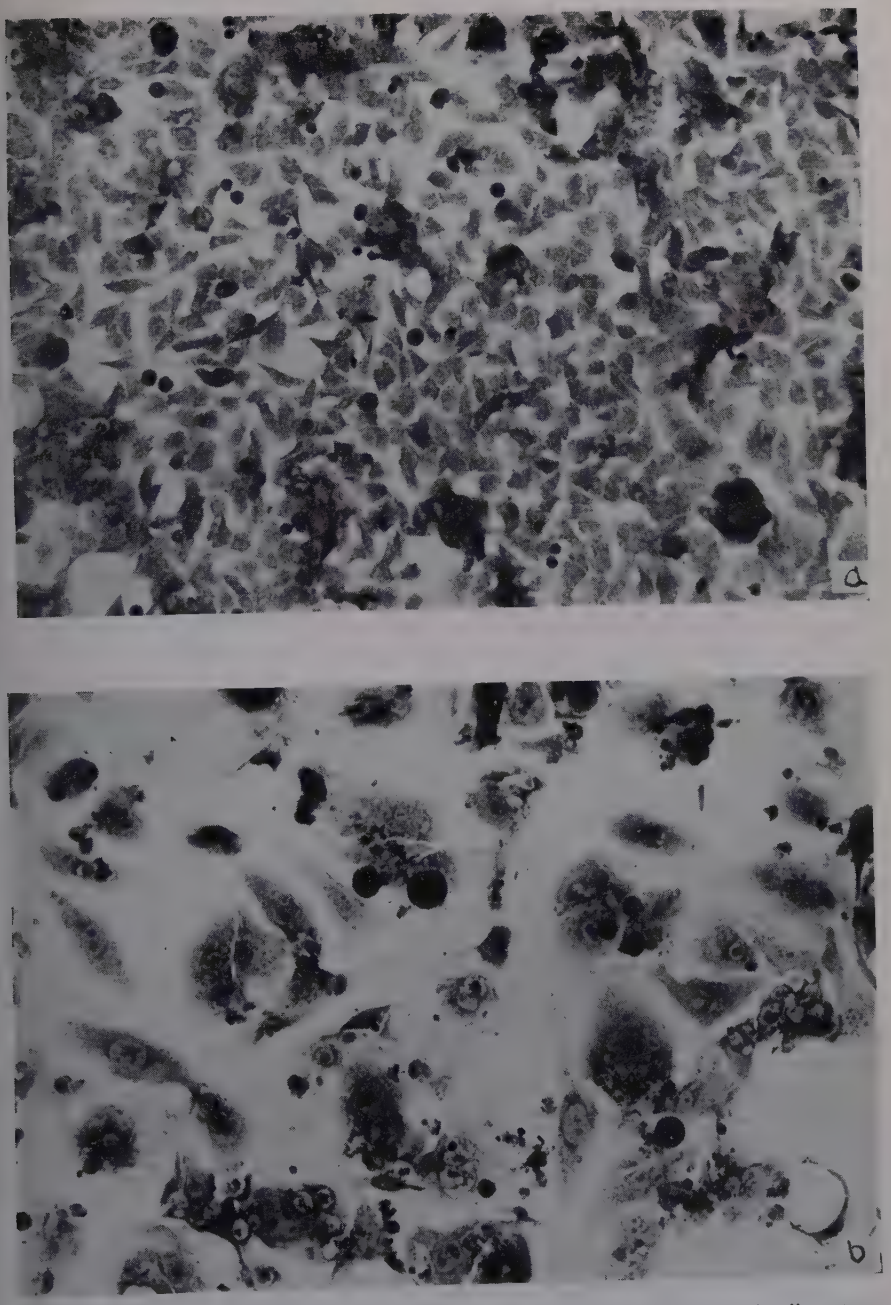


FIGURE 3. (*a*) Control cover slip at 192 hours sample time shows normal cell pattern; culture strain $J111C1_{1559}$; and (*b*) cover slip at 192 hours sample time from culture that differed from (*a*) only in that 1.0 μc. tritiated thymidine per milliliter of medium was added at zero time; cells show marked radiation effect; magnification is the same as (*a*).

factor was so set that near the middle of the slanted cover slips (see FIGURE 4*b*) the gradient factor was right for optimal growth of these cells while at the top and bottom (FIGURE 4*a* and *b* respectively) it was too low and too high. Even at the bottom it was too high for the monocytic series that were also present in the same culture, so that essentially all of the cells on the cover slip were cells of the plasmocytic series. Note that at the bottom of the cover slip while the cells appeared healthy there was no labeling; in other words no

TABLE 1

TRITIUM-LABELED THYMIDINE EXPERIMENT ON CULTURE $J181_{280}J111_{1396}$*

Initial gradient factor	Depth (mm.)	Nuclei/mm.²			Labeled (%)		Mitoses (%)		Mitoses labeled (%)	Dead cells (%)	
		Predicted	6 hr.	24 hr.	6 hr.	24 hr.	6 hr.	24 hr.	24 hr.	6 hr.	24 hr.
0.006	3	60	75	84†	0	19.7	10.7	18.7	56.4	16.3	14.4
0.012	6	120	100	100†	0	19.3	1.6	3.5	36.4	10.6	5.8
0.018	9	180	78	145‡	0	18.4	2.5	6.6	10.0	8.7	9.7
0.028	14	280	54	69‡	0	3.0	0.6	3.3	0.0	17.1	15.9

* Twenty nuclei/mm.³ inoculated.
† All plasmocytic series.
‡ A few cells of monocytic series.

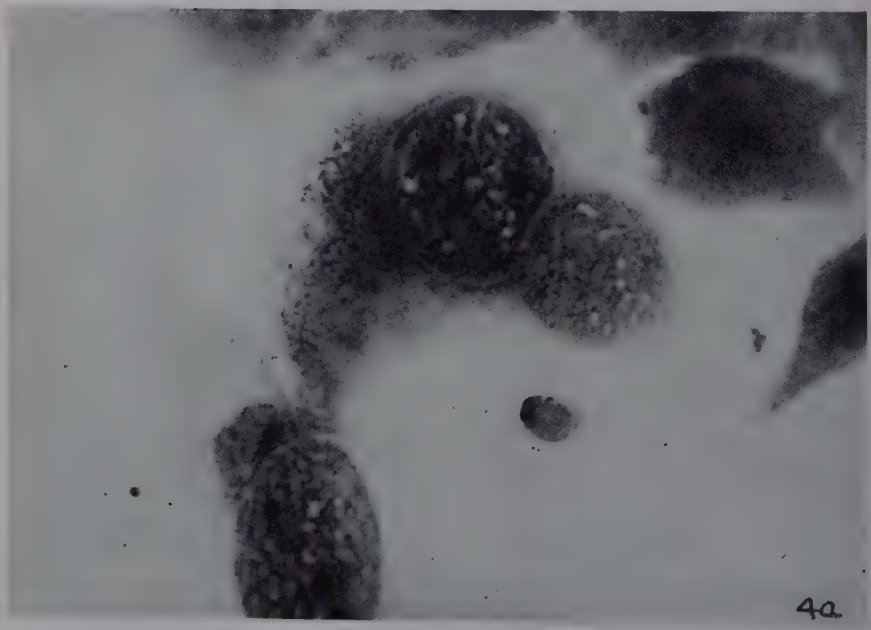
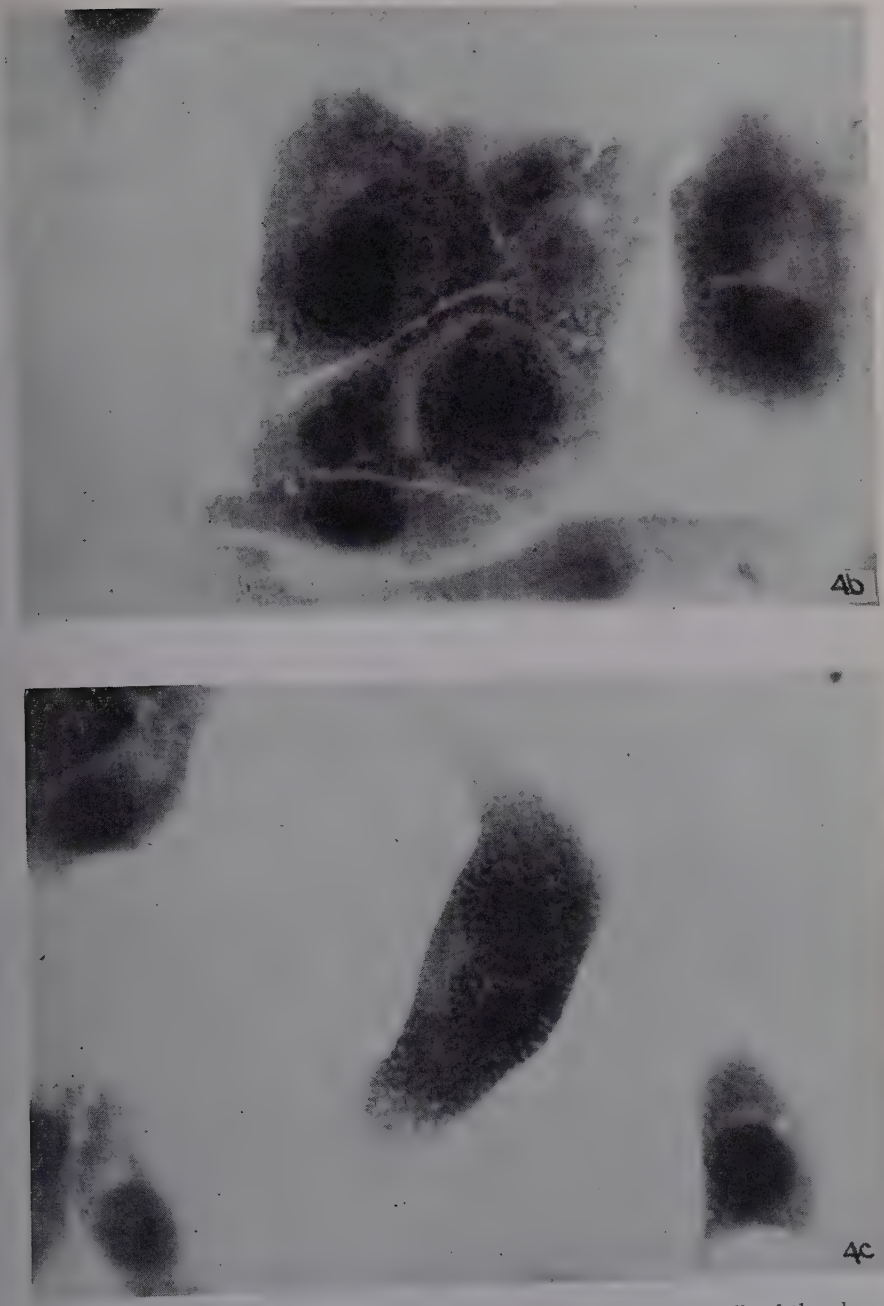


FIGURE 4. (*a*) Appearance of labeled dead cells resembling prophase at the top of the cytic series near the middle of the cover slip; and (*c*) healthy appearing cells but unlabeled



cover slip (see text); (*b*, *see page opposite*) appearance of healthy labeled cells of the plasmo- from near the bottom of the cover slip.

DNA synthesis had occurred. In the middle of the cover slip there was excellent labeling and healthy cell morphology, and at the top of the cover slip there was labeling, but the majority of cells were dead in prophase. This experiment shows that interpretation of any results of irradiation must clearly state the exact conditions under which that irradiation was given and shows the fallacy of depending on mitotic counts unless conditions for cell survival are optimal.

For those who wish to use culture techniques on human hemic cells, 14 strains in culture for 2.5 to nearly 7 years representing all types except the thrombocytic series of human hemic cells are available.[30] Unfortunately, however, evolution goes on in these cells and neither the life span of the n cells, the mitotic rate, nor the chromosome number[37] of these cells bears any relationship to that when they were initially isolated from blood. Since the total alpha-2-alpha divisions in any cell series in man[4,38] are only 4 to 5 in 20 years while as many occur in a few days in culture, change in genetic material of the somatic alpha cell of necessity occurs much more rapidly in cultures than it does in the body. That is why cultured cells so often become malignant.[3]

Summary

Published observations of 30-years study of the effects of ionizing radiation on human hemic cells *in vivo* and *in vitro* are outlined. Heretofore unpublished confirmation of Lajtha's concept of the synthetic period in the middle of the intermitotic period is presented. Further evidence that the effect of any agent either on cells in culture or in the body must be interpreted in the light of the concept of an ecology of cells is illustrated. The way in which the alpha cell-n cell concept of cell division in the multicellular organism clarifies the interpretation and understanding of radiation biology as well as cancer is explained.

Acknowledgments

Nearly everyone employed in the Division of Experimental Medicine and all the residents and fellows since 1930 have participated in these studies; the key investigators are co-authors of the papers cited. However, the major contributors to heretofore unpublished data in this paper are John Brooke. Joseph Tanzer, Donald P. Jenkins, and Russell Lawson.

References

1. Selling, L. & E. E. Osgood. 1938. Action of benzol, roentgen rays, and radioactive substances on the blood and blood-forming tissues. *In* Handbook of Hematology, Section 39. H. Downey, Ed. Hoeber. New York, N.Y.
2. Osgood, E. E. 1959. Regulation of cell proliferation. *In* The Kinetics of Cellular Proliferation. : 282–289. F. Stohlman, Jr., Ed. Grune & Stratton. New York, N.Y.
3. Osgood, E. E. 1957. A unifying concept of the etiology of the leukemias, lymphomas, and cancers. J. Nat. Cancer Inst. **18:** 155–166.
4. Osgood, E. E. 1960. Leukemia as a disturbance of growth regulation. *In* Current Concepts in Leukemia, Report of the Thirty-fourth Ross Conference on Pediatric Research. : 61–69. D. Hammond, Ed. Ross Laboratories. Columbus, Ohio.
5. Osgood, E. E. 1959. Blood cell survival in tissue cultures. Ann. N.Y. Acad. Sci. **77:** 777–796.

6. Osgood, E. E. 1958. Control of peripheral concentration of leukocytes. *In* Symposium on Homeostatic Mechanisms, Vol. 10, Brookhaven Symposia in Biology. : 31–51. Brookhaven National Laboratory. New York, N.Y.
7. Osgood, E. E. 1942. Is the action of roentgen rays direct or indirect? An investigation of this question by the method of human marrow culture. Am. J. Roentgenol. **48:** 214–219.
8. Osgood, E. E., A. J. Seaman & H. Tivey. 1955. Comparative survival times of X-ray treated versus P^{32} treated patients with chronic leukemias under the program of titrated, regularly spaced total-body irradiation. Radiology. **64:** 373–380.
9. Osgood, E. E. & A. J. Seaman. 1952. Treatment of the chronic leukemias: results of therapy of 163 patients by titrated, regularly spaced total body radioactive phosphorus or roentgen irradiation. J. Am. Med. Assoc. **150:** 1372–1379.
10. Osgood, E. E., A. J. Seaman & H. Tivey. 1953. Études radiobiologiques sur les cellules du sang chez l'homme. Rev. hemat. **8:** 403–419.
11. Tivey, H. 1954. The prognosis for survival in chronic granulocytic and lymphocytic leukemia. Am. J. Roentgenol. **72:** 68–93.
12. Osgood, E. E. 1956. Treatment of the leukemias and polycythemia vera with radioactive phosphorus. *In* Therapeutic Use of Artificial Radioisotopes, Chap. 7. P. F. Hahn, Ed. John Wiley & Sons, Inc. New York, N.Y.
13. Osgood, E. E., A. J. Seaman & R. D. Koler. 1957. Natural history and course of the leukemias. *In* Proceedings of the Third National Cancer Conference. : 366–382.
14. Osgood, E. E., A. J. Seaman & R. D. Koler. 1958. The results of a 15-year program of treatment of the chronic leukemias with titrated regularly spaced total body irradiation with P^{32} or X ray. *In* Proceedings of the Sixth International Congress of the International Society of Hematology. : 44–55. Grune & Stratton. New York, N.Y.
15. Osgood, E. E. & G. J. Bracher. 1939. Culture of human marrow; studies of the effects of roentgen rays. Ann. Int. Med. **13:** 563-575.
16. Osgood, E. E. 1939. Marrow cultures. *In* A Symposium on the Blood. Univ. of Wisconsin Press. Madison, Wis.
17. Osgood, E. E. & I. T. Chu. 1950. The effect of nitrogen mustard on granulocytic cells as observed by the marrow culture technique. Cancer Research. **10:** 98–102.
18. Osgood, E. E. 1960. The threshold dose of P^{32} for leukemic cells of the lymphocytic and granulocytic series. Blood. **14:** 1104–1121.
19. Tivey, H. & E. E. Osgood. 1950. The biological half life of radioactive phosphorus in the blood of patients with leukemia. I. Whole blood—with deviations of observations from predicted values and an estimate of total-phosphorus turnover rate. Cancer. **3:** 992–1002.
20. Osgood, E. E. & H. Tivey. 1950. The biological half life of radioactive phosphorus in the blood of patients with leukemia. II. Plasma—with deviations of observations from predicted values and an estimate of total-phosphorus turnover rate. Cancer. **3:** 1003–1009.
21. Tivey, H. & E. E. Osgood. 1950. The biological half life of radioactive phosphorus in the blood of patients with leukemia. III. Erythrocytes—with deviations of observations from predicted values. Cancer. **3:** 1010–1013.
22. Osgood, E. E. & H. Tivey. 1950. The biological half life of radioactive phosphorus in the blood of patients with leukemia. IV. Leukocytes—Radioactive phosphorus content and the relation to plasma-P^{32} levels. Cancer. **3:** 1014–1017.
23. Osgood, E. E. 1951. Titrated, regularly spaced radioactive phosphorus or spray roentgen therapy of leukemias. Arch. Int. Med. **87:** 329–348.
24. Koler, R. D., A. J. Seaman & E. E. Osgood. 1958. Continous therapy of acute leukemias: Comparison of therapeutic agents. *In* Proceedings of the Sixth International Congress of the International Society of Hematology. : 195–206. Grune & Stratton. New York, N.Y.
25. Osgood, E. E. 1959. Polycythemia vera. *In* Current Therapy. : 184–186. W. B. Saunders Co. Philadelphia, Pa.
26. Osgood, E. E., P. C. Aebersold, L. A. Erf & E. A. Packham. 1942. Studies of the effects of million volt roentgen rays, 200 kilovolt roentgen rays, radioactive phosphorus and neutron rays by the marrow culture technique. Am. J. Med. Sci. **204:** 372–381.
27. Osgood, E. E. & I. T. Chu. 1948. The effect of urethane on the nuclear morphology of cells of the granulocyte series. Blood. **3:** 911–917.
28. Lajtha, L. G., R. Oliver, T. Kumatori & T. Ellis. 1958. On the mechanism of radiation effect on DNA synthesis. Radiation Research. **8:** 1–16.
29. Osgood, E. E. 1959. Observations on human hemic cells in long term culture. Haematologica Latina. **2:** 53–63.

30. Brooke, J. H. & E. E. Osgood. 1959. Long-term mixed cultures of human hemic cells with granulocytic, lymphocytic, plasmocytic, and erythrocytic series represented. Blood. **14:** 803–815.
31. Osgood, E. E. & M. L. Krippaehne. 1955. The gradient tissue culture method. Exptl. Cell Research. **9:** 116–127.
32. Osgood, E. E. 1957. Observations on human leukemic cells in culture. *In* Henry Ford Hospital International Symposium, The Leukemias: Etiology, Pathophysiology, and Treatment. : 227–243. J. Rebuck, F. H. Bethell & R. W. Monto, Eds. Academic Press, Inc. New York, N.Y.
33. Rigas, D. A. 1958. Kinetics of isotope incorporation into the deoxyribonucleic acid (DNA) of tissues; life span and generation time of cells. Bull. Math. Biophys. **20:** 33–70.
34. Rigas, D. A. 1959. Dynamics of cell proliferation and isotope incorporation into deoxyribonucleic acid. *In* The Kinetics of Cellular Proliferation. : 408–430. F. Stohlman, Jr., Ed. Grune & Stratton. New York, N.Y.
35. Puck, T. T., P. I. Marcus & S. J. Cierciura. 1956. Clonal growth of mammalian cells *in vitro*; growth characteristics of colonies from single HeLa cells with and without a "feeder" layer. J. Exptl. Med. **103:** 273–283.
36. Rubini, J. R., E. P. Cronkite, V. P. Bond & T. M. Fliedner. 1960. The metabolism and fate of tritiated thymidine in man. J. Clin. Invest. **39:** 909–918.
37. Brooke, J. H., D. P. Jenkins, R. K. Lawson & E. E. Osgood. Chromosome uncoiling and dissociation as affecting the human karyotype. In preparation.
38. Osgood, E. E. 1955. Development and growth of hematopoietic tissues: with a clinically practical method of growth analysis. Pediatrics. **15:** 733–751.

QUALITATIVE AND QUANTITATIVE REACTIONS OF LYMPHOCYTES TO X RAYS*

Robert Schrek

Tumor Research Laboratory, Research Service and Surgical Service, Veterans Administration Hospital, Hines, Ill.

The reactions of the lymphocyte to X rays appear to be unique. It is only the nondividing cell that is killed by small doses of X rays. I shall show later that a dose as small as 10 r produces a perceptible effect on the lymphocyte. This nondividing cell is approximately as sensitive as a cell in mitosis.

On the other hand, there are no unique phenomena in science. If we understood the reactions of lymphocytes to X rays, we should probably understand to a considerable extent the radiosensitivity of dividing cells, and vice versa. In view of the relative ease of studying lymphocytes both qualitatively and quantitatively, I believe that the biochemical and physiologic mechanisms of radiosensitivity will first be understood in lymphocytes. In this paper, I present some data that must be explained in any theory of radiosensitivity.

Radiosensitivity of Human Lymphocytes

The qualitative or cytologic reactions of the lymphocyte can be best studied and presented by phase-contrast microscopy and time-lapse cinemicrography.[1] The cells in 50 per cent homologous serum were incubated and photographed in a special slide chamber.[2] Study of the time-lapse motion picture shows that X rays produced 2 distinct types of death in lymphocytes. After a large dose of irradiation (4000 r or more) human lymphocytes rounded up and became completely motionless in a few hours.[3] In addition there was an increase in contrast of cytologic structures, for example, cell and nuclear walls, cytoplasmic granules, and chromatin masses. The cells were undoubtedly dead, and this type of death may be called death by delayed fixation. The dead cells underwent secondary post-mortem or autolytic changes, including shrinkage of nucleus and thickening and blurring of chromatin masses. These changes finally resulted in a small dark nucleus showing little or no structure.

After irradiation with 1000 r, human lymphocytes usually remained normal for about 12 hours. Then one cell after another developed one or more intranuclear vacuoles usually associated with rapid changes in the shape of the cell and the nucleus. The vacuoles were surrounded by a thick ring of chromatin. Usually the intranuclear vacuoles ruptured and the chromatin ring condensed into a small, round, dark, structureless, pyknotic, and sometimes fragmented, nucleus. This type of cell death is called death by intranuclear vacuolization.

In 2 days nearly all the lymphocytes irradiated with 1000 r had died by the process of intranuclear vacuolization. In contrast, the nonirradiated lymphocytes were nearly all motile and obviously alive. After incubation in the slide chamber for 6 to 10 days, the nonirradiated cells were seen to undergo cell death. Surprisingly enough, the nonirradiated lymphocytes also died by the

* The work reported in this paper was supported in part by Research Grant CY-2476 from the National Cancer Institute, Public Health Service, Bethesda, Md.

process of intranuclear vacuolization with the production of pyknotic, fragmented nuclei. In other words, the nonirradiated lymphocytes died by the same process as lymphocytes irradiated with moderate doses of X rays. Apparently moderate doses of irradiation did not kill lymphocytes but only accelerated normal death. This conclusion is based on the assumption that lymphocytes in the slide chamber die by a normal physiologic process.

Qualitative data are, however, not enough. Fortunately, the slide-chamber method used for photography could also be used to obtain quantitative data. The time-lapse cinemicrographic findings permit us to define and recognize viable lymphocytes. These are round, elongated, or hand mirror-shaped and have a nucleus with light gray chromatin masses. Dead lymphocytes are nearly always perfectly round with dark, pyknotic, structureless nuclei. Dying lymphocytes with irregular shape and vacuolated nuclei were included in the count of the dead lymphocytes. After large doses of X rays, dead lymphocytes had "fixed" or autolyzed nuclei.

The number of viable lymphocytes were counted daily in a strip 10 x 0.04 millimeter in area. Survival curves were then constructed with the original count as 100 per cent. The survival curve was summarized by calculating the 10 per cent survival time (ST). The effect produced by irradiation was measured by the formula:

$$\text{Effect} = 100\left(1 - \frac{\text{10 per cent ST of irradiated cells}}{\text{10 per cent ST of nonirradiated cells}}\right)$$

It should be noted that the effect is measured by an arbitrary index.

The effect of varying doses of X rays on human lymphocytes from lymph nodes and from blood is presented in the scattergram of FIGURE 1. The abscissa of the graph represents the logarithm of the dose and the ordinate, the effect. Inspection of the scattergram shows that lymphocytes from the blood had approximately the same radiosensitivity as lymphocytes derived from lymph nodes.

What is the minimum dose of irradiation that produces an effect? FIGURE 1 shows that a dosage of 10 r produced a consistent effect with an average of 40.9 per cent and a standard error of 2.2 per cent. The effect produced by 10 r is statistically significant. Dosages less than 10 r produced inconsistent effects. It should be noted that no formal experiments were set up to determine the minimum dosage. The experimental error in this work was such that it was not possible to determine with confidence a 10 per cent effect. Such an effect could, I believe, be measured by a moderate increase in the number of slide-chambers used and in the number of cells counted.

The data in FIGURE 1 can be expressed as a linear regression equation. The formula for the equation is:

$$\text{Effect} = 12.8 + 25.2 \log (\text{dose})$$

The linear curve representing the equation is shown in the figure. A 50 per cent effect was produced by 30 r. If this line in FIGURE 1 is extrapolated, we find that a dosage of 0.7 r produces an effect of 0. This analysis suggests the possibility of a threshold dose for the radiosensitivity of lymphocytes.

FIGURE 1 and the regression equation demonstrate the linear relationship between effect and logarithm of dose. This finding has an interesting analogy in photography. To standardize photographic film, it is exposed to various intensities of light and developed under standard conditions. The density is then measured and graphed, as in FIGURE 2, against the logarithm of the exposure. It is seen that most of the data can be represented by a linear curve. With photographic film, there is then a linear relationship between density and

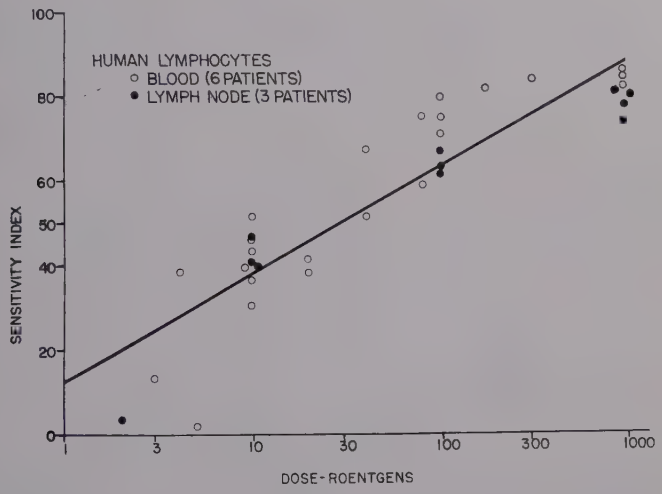


FIGURE 1. Scattergram of the effect of irradiation on human lymphocytes from blood and lymph nodes of nonleukemic patients. The abscissa represents the logarithm of the dose of X rays; the ordinate represents the effect or sensitivity index, as measured by the formula given in the text. The linear curve represents the regression equation:

$$\text{Effect} = 12.8 + 25.2 \log (\text{dose})$$

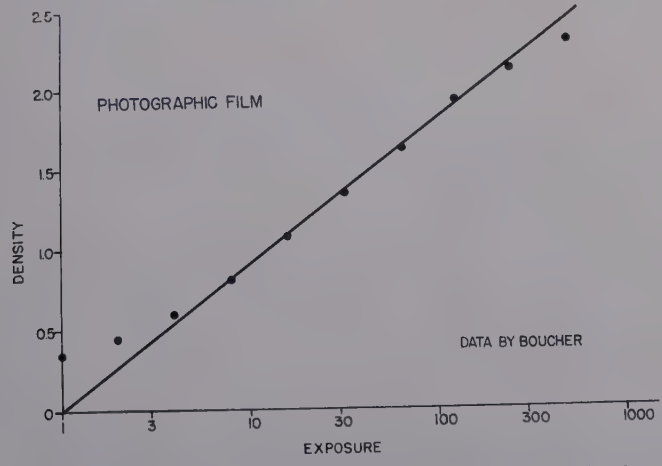


FIGURE 2. Effect of exposure in density of photographic film after development. Data are obtained from Boucher.[9]

the logarithm of exposure. Similarly with human lymphocytes, there is a linear relationship between effect and logarithm of dose of X rays.

Radiosensitivity of Lymphocytes of Lower Animals

Lymphocytes from the blood of rats and other species were suspended in 50 per cent homologous serum and studied by the slide-chamber method. It is

TABLE 1

IN VITRO 10 PER CENT SURVIVAL TIME OF LYMPHOCYTES FROM BLOOD OF DIFFERENT SPECIES

Blood lymphocytes from	No.	10% ST (days)	
		Mean	Range
Man	6	9.7	7.2–14.7
Rabbit	2	2.6	2.4– 2.7
Rat	5	2.1	1.6– 2.7
Guinea pig	4	1.5	1.1– 1.8

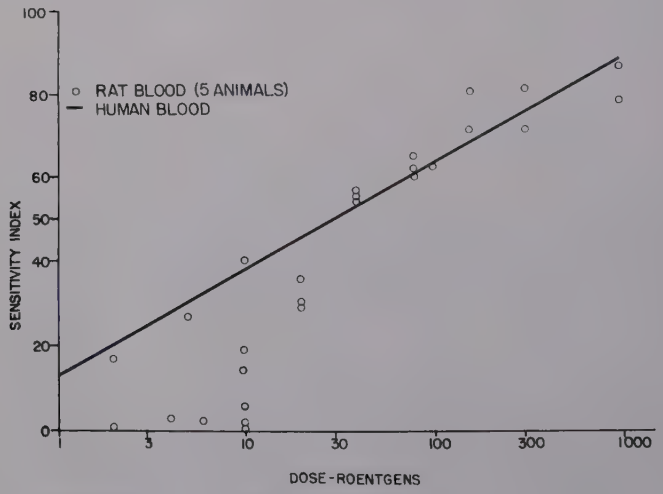


FIGURE 3. Scattergram of the effect of irradiation on lymphocytes from rat blood. Abscissa and ordinate are the same as in FIGURE 1. The linear curve is the regression equation for human lymphocytes, as in FIGURE 1.

seen in TABLE 1 that untreated lymphocytes of different species differ in their 10 per cent survival time. The blood lymphocytes of the guinea pig had the shortest 10 per cent survival time (1.5 days); human blood lymphocytes, the longest (9.7 days). This variability in the survival time of untreated lymphocytes of different species complicates the measurement of radiosensitivity as there is no theoretically valid method for its measurement. The arbitrary formula used for measuring the effect of irradiation takes into consideration the variation in the survival of untreated lymphocytes. FIGURE 3 shows a scattergram for the radiosensitivity of blood lymphocytes from 5 rats. The

linear curve in FIGURE 3 represents the sensitivity of human lymphocytes as determined in FIGURE 1. Rat lymphocytes had, it seems, a slightly lesser radiosensitivity than the human lymphocytes.

A similar graph for rabbit cells (FIGURE 4) shows that lymphocytes of the thymus and of blood had approximately the same radiosensitivity. The lymphocytes of the rabbit, like those of the rat, were apparently less radiosensitive than those of man.

The lymphocytes from the blood of guinea pigs have a relatively short 10 per cent survival time (1.5 days, TABLE 1). It was not feasible to obtain counts on the cells more than twice a day. As a result relatively few counts could be made during the survival period of lymphocytes of the guinea pig. A rela-

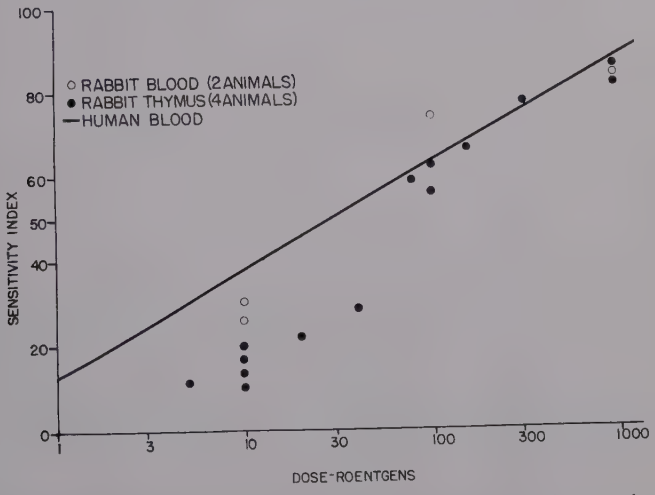


FIGURE 4. Scattergram of the effect of irradiation on rabbit lymphocytes from the blood and the thymus. Abscissa and ordinate are the same as in FIGURE 1. The linear curve is the regression equation for human lymphocytes, as in FIGURE 1.

tively large experimental error was therefore obtained in measuring the 10 per cent survival time and the effect produced by irradiation.

The dose effect curve obtained on 4 animals is presented in FIGURE 5. The results in this graph are more variable than those obtained with other animals due to the larger experimental error. On comparison with the curve representing human lymphocytes, it is seen that the effect produced by irradiation of guinea pig lymphocytes is consistently less than that for human lymphocytes. The guinea pig lymphocytes seemed less sensitive than human and rat lymphocytes. This finding is surprising since, *in vivo*, the guinea pigs are more radiosensitive than rats; the LD_{50} in 30 days is 200 to 400 r for guinea pigs and 600 to 700 r for rats.[4]

Another analysis of radiosensitivity is given in TABLE 2, which compares the effect of irradiation with 10 r on the lymphocytes of the 4 species. According to statistical analysis, the irradiation had a significantly greater effect on human lymphocytes than on the lymphocytes of the lower animals. This finding may

however be an artifact and may be dependent on the arbitrary definition of effect and on the relatively prolonged survival time of nonirradiated human lymphocytes.

Factors Affecting Radiosensitivity

The standard procedures used in previous experiments were designed to provide more or less physiologic conditions. The medium consisted of equal

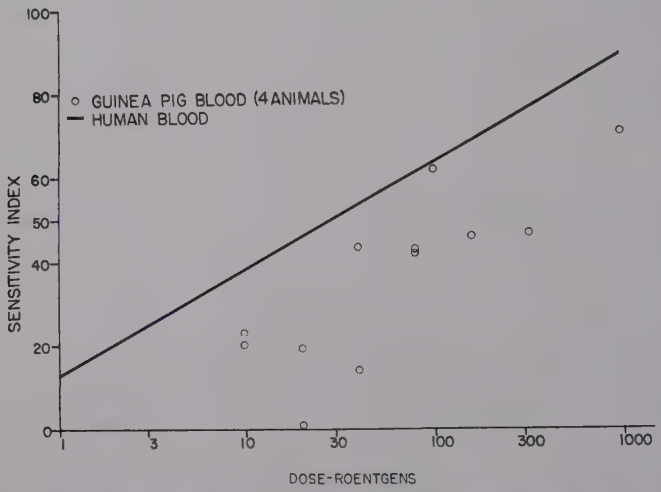


FIGURE 5. Scattergram of the effect of irradiation on lymphocytes from guinea pig blood Abscissa and ordinate are the same as in FIGURE 1. The linear curve is the regression equation for human lymphocytes, as in FIGURE 1.

TABLE 2

EFFECT OF IRRADIATION WITH 10 R ON LYMPHOCYTES FROM BLOOD OF DIFFERENT SPECIES

Blood lymphocytes from	No.	Effect of 10 r	
		Mean	Range
Man	8	40.9	30.5 to 51.6
Rabbit	6	19.5	10.3 to 30.6
Rat	5	12.4	−6.3 to 40.5
Guinea pig	2	21.7	20.2 to 23.2

parts of normal homologous serum and tissue culture medium TC 199. The *p*H of the suspension was approximately 7.8, which declined slightly during incubation as indicated by the color of the added phenol red. The initial oxidation-reduction potential of the suspension was high as indicated by a bluish color when the dye, 2,6 dichlorophenol indophenol, was added. During the course of incubation of human lymphocytes the dye showed a slight decrease in color but was not decolorized completely. The incubation temperature was 37° C. ± 0.2° C. in the incubator and ±1° C. during cell counting.

The effect of various factors on radiosensitivity was tested not by the slide-chamber method but by the unstained cell-count method used previously.[5] The cells used in those studies were lymphocytes from the rabbit thymus. It is necessary to consider separately the conditions during the period of irradiation and the conditions following irradiation.

TABLE 3 summarizes some findings on the effect of various factors on radiosensitivity. Decreasing the temperature of the suspension even to 4° C. during irradiation had no significant effect on radiosensitivity.[5] Decreasing the oxygen tension by packing the cells during irradiation resulted in decreased radiosensitivity; under these conditions the dose of X rays had to be about double to give the same radiation effect.[6]

Similarly many other types of cells show decreased radiosensitivity if they are irradiated under anoxic conditions. Addition of cysteine (15 m*M*) before irradiation also decreased radiosensitivity by a factor of 2.[7] The effect pro-

TABLE 3

FACTORS THAT AFFECT THE RADIOSENSITIVITY OF LYMPHOCYTES FROM RABBIT THYMUS, ACCORDING TO UNSTAINED CELL COUNTS

	Factors that produce		
	Standard sensitivity	Decreased radiosensitivity	No radiosensitivity
During or before irradiation			
O_2 tension:	4–37°	Low	
Temperature C.:			
Cysteine:		15 m*M* at 37° C.	
After irradiation			
Temperature C.:	37°	21–27°; 41°	17°; 45°
*p*H:	7.6	6.5	6.0

duced by cysteine was not, however, simply due to a reduction of oxygen tension. The cysteine and the cells had to be in contact for at least 15 min. at a temperature of 37° C. to produce the decreased radiosensitivity. This finding would suggest that the decreased radiosensitivity is the result of a chemical or metabolic interaction of the cells with the cysteine.

It has been seen that the temperature of the suspension during irradiation had no significant effect on radiosensitivity. In contrast the temperature of the suspension after irradiation did affect the response of the cells to the irradiation. Lymphocytes irradiated at 1000 r and incubated at 45° C. or at 17° C. survived as long as nonirradiated cells. In other words the lymphocytes were not radiosensitive when incubated at 45° C. or 17° C. Nonirradiated lymphocytes at 17° C. survived longer than those at 37° C.; the 50 per cent survival was 7.2 days at 17° C. and 0.9 days at 37° C. Furthermore lymphocytes incubated at 17° C. did not die by intranuclear vacuolization. Instead they died apparently without any major change in cytologic structures. We have seen previously that lymphocytes incubated at 37° C. developed intranuclear vacuoles and that X rays accelerated the development of these vacuoles. In contrast, lymphocytes incubated at 17° C. did not develop intranuclear vac-

uoles. Therefore it would seem that the radioresistance of lymphocytes at 17° C. was correlated with the failure of these cells to undergo intranuclear vacuolization.

TABLE 3 also shows the effect of *p*H on radiosensitivity. Addition of NaOH to *p*H 8.5 had little effect on the survival time of nonirradiated lymphocytes and had no definite effect on radiosensitivity. Addition of HCl and other acids to *p*H 6.0 also had no effect on the survival time of the nonirradiated lymphocytes. The lymphocytes of the acidified suspension showed a decreased radiosensitivity and at *p*H 6.0, the lymphocytes were resistant to irradiation with 1000 r.

It should be noted that the standard conditions used in the unstained cell count method were designed to be more or less physiologic. Any deviation from these conditions resulted either in no change or in a decrease of radiosensitivity of the lymphocytes. No factor was found which increased radiosensitivity.

TABLE 4

IN VITRO 10 PER CENT SURVIVAL TIME OF BLOOD LYMPHOCYTES FROM RATS IRRADIATED *IN VITRO* AND FROM CONTROL RATS

Dose	Time between X ray and bleeding	No. of rats	10% ST (days)	
			Mean	Range
0 r		7	2.33	1.5–3.0
100 r	15 min.	2	0.67	0.6–0.7
50 r	15 min.	8	0.75	0.3–1.2
50 r	1 day	1	1.46	
50 r	2 days	1	1.23	
25 r	15 min.	5	2.07	1.8–2.4

Radiosensitivity of Circulating Lymphocytes in Vivo

The question arises: Do the *in vitro* findings have any significance on radiation *in vivo*? More specifically the problem is: Does radiation of an animal destroy lymphocytes in the circulating blood? The prevalent concept has been, and probably still is, that irradiation *in vivo* inhibits lymphocyte formation but has no effect on lymphocyte destruction. Theoretical considerations from *in vitro* findings would suggest that circulating lymphocytes are sensitive to irradiation *in vivo*. Furthermore, quantitative data and mathematical analysis convinced Hulse[8] that irradiation *in vivo* caused first an excessive destruction and secondarily an inhibition of formation of lymphocytes.

The slide-chamber method provides a direct approach to the problem of the effect of irradiation *in vivo*. Rats were subjected to total body irradiation and were bled within 15 min. The blood lymphocytes of irradiated and control rats were concentrated, suspended in 50 per cent normal rat serum, and incubated in slide chambers. The viable lymphocytes were counted twice a day and the 10 per cent survival time determined. The results are shown in TABLE 4. It is seen that lymphocytes from irradiated rats had a relatively short survival time as compared to lymphocytes from nonirradiated animals. Ir-

radiation of the rats with 50 r produced a significant decrease in the 10 per cent survival time. Blood drawn 24 and 48 hours after irradiation of rats with 50 r also contained physiologically injured lymphocytes, as indicated by a shortened 10 per cent survival time *in vitro*. The findings indicate that irradiation *in vivo* produced a deleterious change in the circulating lymphocytes. This deleterious change was demonstrable as a decreased survival time *in vitro* and would have, presumably, caused a shortened life span of the lymphocytes *in vivo*.

The last problem I propose to consider is: What do the *in vitro* findings mean in regard to the mechanism of the radiosensitivity of lymphocytes? It has been seen that the maximum radiosensitivity occurred when the lymphocytes were under physiologic conditions. Furthermore, deviation from these conditions, such as *p*H 6.0 or incubation at 17° C., resulted in resistance of the cells to irradiation with 1000 r. These findings suggest that the radiosensitivity is due to a specific metabolic process in lymphocytes. It should be noted, further, that nondividing lymphocytes have approximately the same order of radiosensitivity as many cells in mitotic division. It would seem that a specific, radiosensitive metabolic process is present both in lymphocytes and in cells undergoing mitotic division.

Summary

The effect of X rays on lymphocytes of man and lower animals was studied by the slide-chamber method and the method of unstained cell counts. Large doses of X rays (4000 r or more) killed lymphocytes by delayed fixation. Moderate doses (1000 r or less) killed lymphocytes by intranuclear vacuolization. Nonirradiated lymphocytes also died by intranuclear vacuolization, but at a slower rate. Irradiation with 10 r produced a perceptible decrease in the survival time of human lymphocytes incubated at 37° C. in slide chambers. Human lymphocytes were slightly more radiosensitive than lymphocytes of the rat, rabbit, and guinea pig. The effect produced by irradiation was a linear function of the logarithm of the dose.

Blood lymphocytes from rats irradiated *in vivo* with 50 r had a shortened 10 per cent survival time: 0.75 days as compared to 2.3 days for lymphocytes from nonirradiated rats. This shortened survival time demonstrated that irradiation *in vivo* produced a deleterious change in circulating lymphocytes of rats.

References

1. Schrek, R. 1955. Cinemicrographic observations and theoretical considerations on reactions of lymphocytes to X rays. Radiology. **65:** 912–919.
2. Schrek, R. 1958. Slide-chamber method to measure sensitivity of cells to toxic agents. AMA Arch. Pathol. **66:** 569–576.
3. Schrek, R. 1957. Dual morphologic reactions of rabbit lymphocytes to X rays. AMA Arch. Pathol. **63:** 252–259.
4. Patt, H. M. & A. M. Brues. 1954. The pathological physiology of radiation injury in the mammal. 1. Physical and biological factors in radiation action. *In* Radiation Biology. **1:** 919–958. A. Hollaender, Ed. McGraw-Hill. New York, N.Y.
5. Schrek, R. 1946. Studies *in vitro* on the physiology of cells. Factors affecting the delayed cytocidal action of X rays. J. Cell. Comp. Physiol. **28:** 277–304.

6. Patt, H. M., M. E. Blackford & R. L. Straube. 1952. Effect of X rays on thymocytes and its modification by cysteine. Proc. Soc. Exptl. Biol. Med. **80:** 92–97.
7. Patt, H. M. 1953. Protective mechanisms in ionizing radiation injury. Physiol. Rev. **33:** 35–75.
8. Hulse, E. V. 1959. Lymphocyte depletion of the blood and bone marrow of the irradiated rat: A quantitative study. Brit. J. Haematol. **5:** 278–283.
9. Boucher, Paul E. 1947. Fundamentals of Photography with Laboratory Experiments. 2nd Ed. Van Nostrand Co., Inc. New York, N.Y.

CYTOCIDAL EFFECTS OF RADIATION ON ORGAN CULTURES

O. A. Trowell

Medical Research Council Radiobiological Unit, Harwell, Berkshire, England

Introduction

The action of ionizing radiation on cells growing in tissue culture has been investigated since the dawn of radiobiology. This early work yielded important information about the effects of radiation on the mitotic process, but it also gave rise to the mistaken belief that cell death after irradiation is always due to mitotic derangement. Furthermore, the cells involved, growing in a plasma clot or on a glass face, were far removed from their natural habitation: they were either embryonic or dedifferentiated, and they were of doubtful ancestry.

The more recent method of *organ* culture now enables us to study the effects of radiation on precisely identified adult cells in their normal tissue location within a few hours of removal from the body and, usually, in the absence of mitosis. The present paper will deal with the acute lethal effects of radiation on the specifically radiosensitive cell types to be found in certain organs.

Our objectives were (1) to measure the innate radiosensitivity of the cell types with some accuracy, (2) to measure such changes in radiosensitivity as may be induced by altering the cell environment either by manipulation of physiological components such as oxygen and *p*H or by addition of protective or sensitizing chemicals, and (3), using organs at different stages of development, to study the changes in radiosensitivity which accompany cell differentiation. For these purposes it is desirable, if not essential, to use organs maintained *in vitro*, for only thus can influences from other parts of the body be excluded and the cell environment be accurately controlled. Furthermore, during the period of irradiation the cell environment can, if necessary, be set widely outside ordinary physiological limits and this may yield valuable information unobtainable in the whole animal.

In most cases the acute lethal effect begins to appear about 2 hours after irradiation and is complete within 24 hours, so it is only necessary to maintain the organs *in vitro* for a few days, and a simple organ-culture technique has proved suitable. It is better to irradiate the cultures rather soon after they have been set up, for example within 24 hours, as radiosensitivity may slowly change *in vitro*. The chief limitation is that the cultures must not exceed about 2 mm. diameter (otherwise the oxygen supply is inadequate) and so only very small organs, such as thyroid, adrenal, pituitary, ovary, eye, and lymph nodes of rats or mice can be cultured whole; larger organs must be cut into pieces of suitable size. For this work it is necessary to use a fluid culture medium, preferably an artificial one of known composition, and to have the gas phase accurately controlled. The organ-culture method designed to meet these requirements will now be described.

Organ-Culture Method

The fluid culture medium is contained in a shallow glass dish, which can be made from a standard 48-mm. outside-diameter capsule (crystallizing dish)

by grinding down the side wall to an outside height of 10 mm. In the dish stands a grid made from perforated metal sheet; the top is 25 × 25 mm., and the edges are bent over to form "legs" 4 mm. high (FIGURE 1). The metal is titanium, and it is used in the form of "expanded metal" sheet, 1.5 mm. mesh, 0.003 inch thick.* Titanium is corrosion-resistant and nontoxic. It can be cleaned by boiling in NaOH and then standing in pure A.R. (not fuming) HNO_3. On top of the grid is laid a sheet of agar 25 × 25 mm. and 1 mm. thick. This is made by pouring the requisite volume of molten agar [2 per cent Agar (Davis) in 0.7 per cent NaCl] into a stainless steel tray of known area and, when cool, cutting out squares with a knife.

The cultures, up to 20 in number, are then planted on the agar, as shown in FIGURE 2. In this figure the culture dish is made of fused silica, which can be used if one is concerned about chemical contamination arising from glass. The organs seen in FIGURE 2 are various (a "mixed grill"), and they can be identified from the caption. The volume of medium (6 ml.) is such that the lower surface of the agar is in contact with medium but the upper surface is 1 to 2 mm. higher than the surrounding fluid level. There is therefore no free fluid moving over the surface of the agar, and the cultures sit on a moist agar base with about two thirds of their surface in direct contact with the gas phase, as may be seen in FIGURE 2.

The culture dish is now placed in a gas-tight culture chamber made of Perspex (Lucite). An opened, empty chamber is shown in FIGURE 3. It is 3 inches in diameter; the lower portion is 1¼ inches high and has a central well 2 inches in diameter and five eighths inch deep; the lid, one half inch thick, is held on by 4 bolts, and the joint is greased with lanoline. Two stainless steel tubes, of one sixteenth inch bore, penetrate the side walls to provide for the passage of gas. In making the chamber, these tubes are driven into slightly undersized holes, and the resulting joint is firm and gas-tight. Normally the gas phase is 95 per cent O_2 and 5 per cent CO_2, and the chambers are regassed morning and evening. The chambers can be kept in a water bath, but it is more convenient to keep them, together with the gas lines and gas cylinders, in a small room maintained at 37° C. The complete assembly is shown in FIGURE 4.

The culture medium normally used is a simple artificial one called T8. It contains the usual Tyrode salts, glucose, insulin, 12 amino acids, thiamine, *p*-aminobenzoic acid, chloramphenicol (Chloromycetin) as antibiotic, and phenol red as indicator.

Many but not all organs can be maintained in a satisfactory state of preservation (judged histologically) for about one week; there is no dedifferentiation and only a very small outgrowth of cells. Details of the behavior of the different organs and of the medium T8 have been given in an earlier paper (Trowell, 1959). The theoretical basis of the method has also been discussed (Trowell, 1961*a*).

Irradiation of Cultures

A 250 Kvp radiotherapy-type X-ray machine was used, and the HVL was 1.2 mm. Cu. The culture chamber was held in a carrier attached to the

* The Expanded Metal Co., Ltd., London, England.

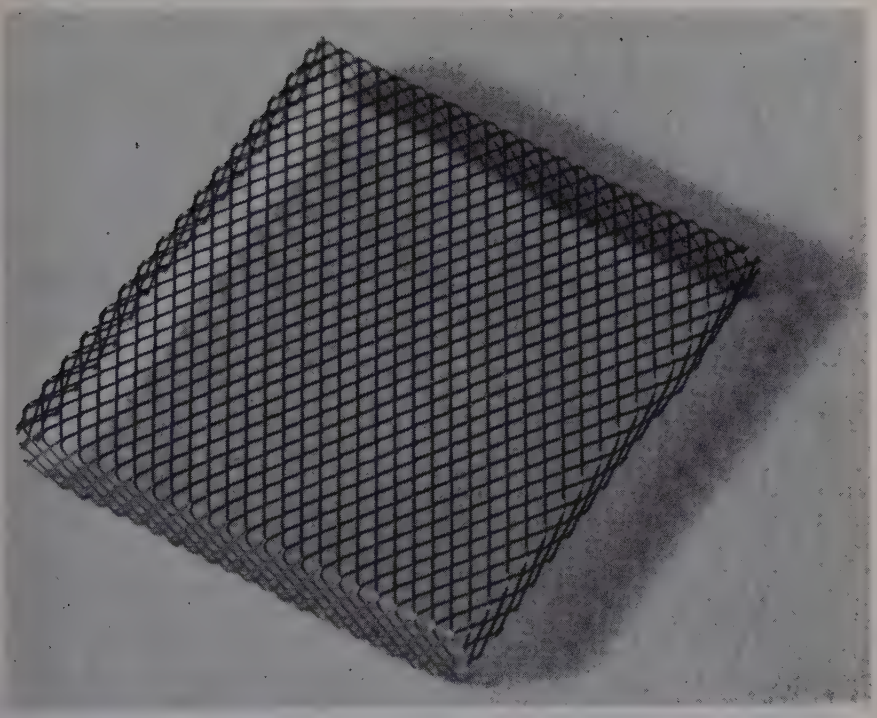


FIGURE 1. Culture grid made of titanium "expanded metal."

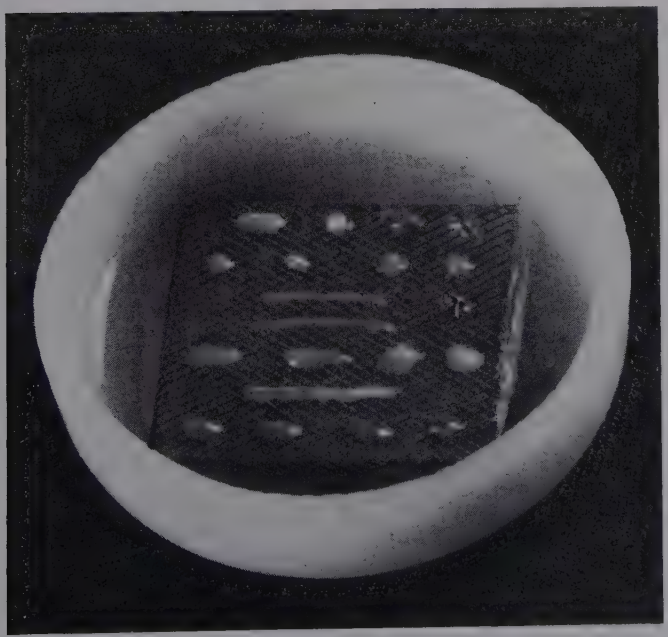


FIGURE 2. Culture dish (silica) containing medium, grid and various organs from young rats. The organs are: row 1 (top), pituitary, pineal, 2 half thyroids; row 2, 2 adrenals, 2 ovaries; row 3, 2 ureters, pin's head (for size); row 4, 2 lymph nodes, two quarter prostates; row 5, abdominal aorta; row 6, 2 pieces of liver, 2 pieces of lung.

FIGURE 3. Culture chamber, empty and opened. There is a filter paper on the floor of the chamber to aid visibility. Chamber is made of Perspex (Lucite), two stainless steel tubes and four stainless steel bolts: the latter not shown.

FIGURE 4. Culture chamber assembled. A glass culture dish and twenty lymph node cultures are shown.

X-ray tube so that its position in the beam and its distance from the anode were always the same (FIGURE 5). The chambers were carried to and from the X-ray machine in a small 37° C. water bath and were kept in another 37° C.

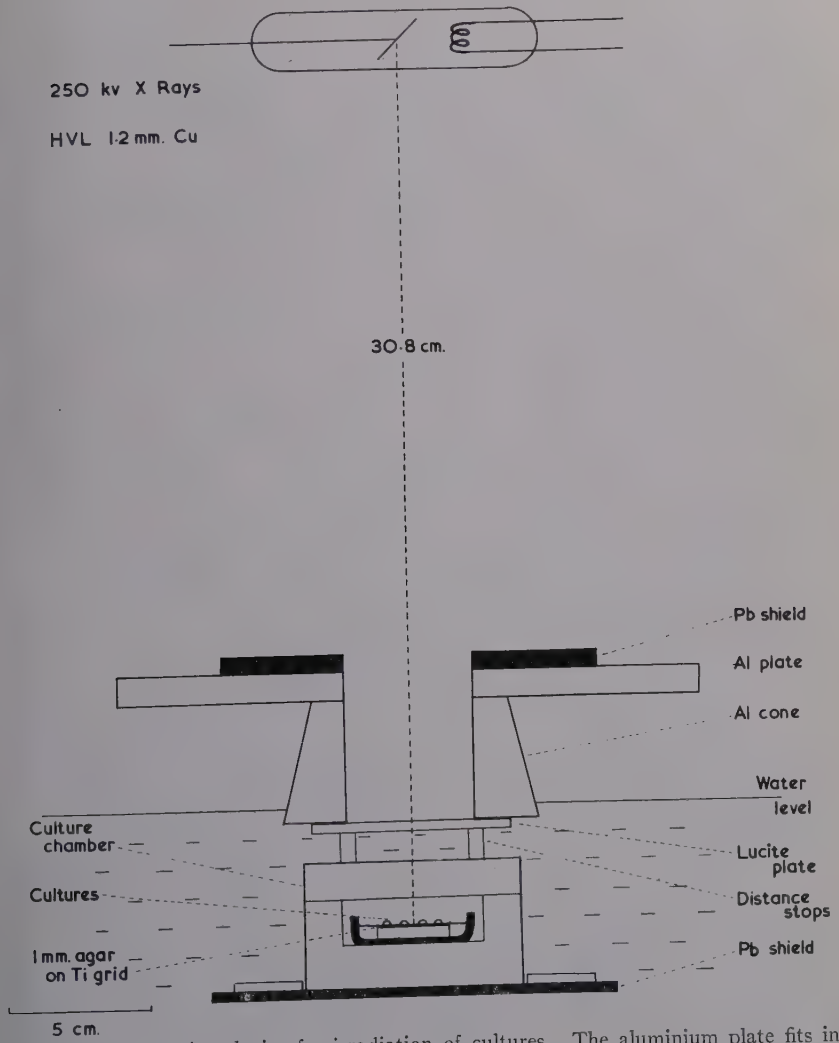


FIGURE 5. Locating device for irradiation of cultures. The aluminium plate fits into the diaphragm holder of the X-ray machine.

bath during irradiation. The dose rate was measured by an M.R.C. Type B.D.2 ionization chamber in an empty culture chamber and in the plane normally occupied by the cultures. The dose rate used was either 141 or 317 rpm, depending on the sensitivity of the organ. Had the cultures been closer to the metal grid a small dose error would have arisen due to secondary electrons arising from the metal. This is avoided here by spacing the cultures

1 mm. away by means of the agar and by using titanium, which has a low atomic number (22).

Measurement of Radiation Damage

The cultures in most cases can be produced rather uniformly; they are maintained under standard conditions, and the dose delivered is accurate to within ±2 per cent. The remaining problem is to measure the radiation effect. We must decide exactly what end point to measure and then how to measure it. This looks straightforward enough but it has proved to be the most troublesome part of the whole project. The over-all error of the experiment turns almost entirely on the accuracy of this measurement. Furthermore, as will be shown later, the interpretation and significance of the results may depend upon the particular end point chosen.

We are here dealing with the outright killing of cells, usually one particular (radiosensitive) cell type in an organ containing many cell types, and we desire to know the percentage of the sensitive type killed by radiation. The percentage of dead cells can be determined at any chosen time after irradiation but, if possible, we want to know the "final percentage killed," which is the percentage that have died when the radiation effect is all over. In radiobiology it is more common to record the "percentage survival," which is obtained by subtracting the final percentage killed from 100.

The cultures must be sacrificed at some determined time after irradiation; they are either fixed and sectioned or else reduced to a suspension of cells or nuclei. Some method of cell counting must now be applied to this material and two clear-cut alternatives present themselves which, for simplicity, will be called (1) dead cell counting and (2) live cell counting.

Dead cell counting. The dead cells are recognized by nuclear pyknosis in fixed preparations or by eosin stainability in fresh preparations. The ratio of dead to living cells (of the radiosensitive type) is determined by counting and this gives the percentage of dead cells present. It would seem that if the determination were made after the radiation effect was all over, the percentage of dead cells found would represent the final percentage killed, but unfortunately this is not the case. Some of the cells die soon after irradiation, others later and, if counting is delayed until the latest ones have just died, by now some of the earlier ones will have disappeared altogether, by autolysis or phagocytic removal. So the total number of cells that have died can never be found. The situation is shown graphically in FIGURE 6, where curve A represents the total cells that have died and curve B the number of dead cells actually present, at time intervals after irradiation. If cell counting is delayed until the radiation effect is complete, as at time T_2, the percentage of dead cells present is 70, and this can be determined by counting. However the true percentage of cells killed is about 80, 10 per cent having died and disappeared, and this fraction that disappears cannot be found by the dead cell counting method.

In practice the counts are usually made at an earlier time, as at T_1, because recently dead cells are more easily recognized, and it must be accepted that the figure obtained, though presumably a measure of radiosensitivity, does

not correspond to the final percentage killed. In certain circumstances it may not even be a valid measure of radiosensitivity, as will be shown later. It is unfortunate that the figures obtained by this dead-cell counting should be of limited value, for the counting can be very accurate, no total counts are needed, and no special control cultures are required.

Live cell counting. The cultures are examined when the radiation effect is over (that is, when no more cells are dying) and the total number of healthy cells (of the radiosensitive type) is counted, which is fairly easy to do. The difficulty is that the total number present before irradiation can never be

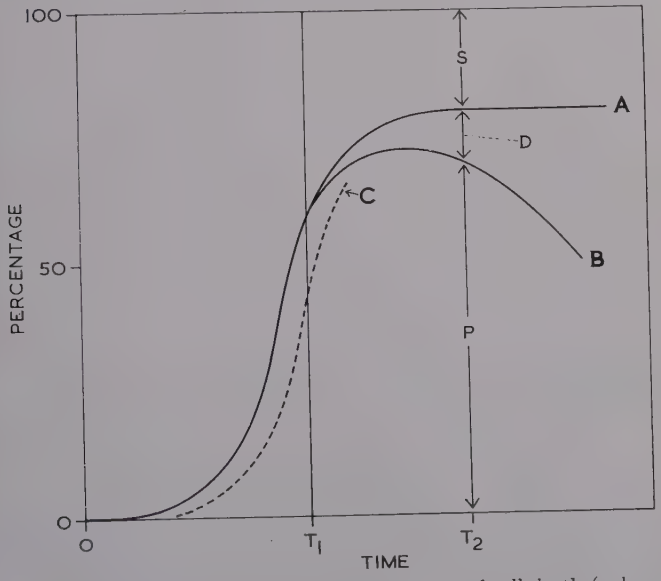


FIGURE 6. Schematic representation of the time course of cell death (pyknosis) and cell disappearance in a population of cells irradiated at time 0. Ordinate is percentage of the original population. Curve *A* = percentage killed, curve *B* = percentage dead (pyknotic) and still present. At time T_2, P = percentage pyknotic, D = percentage dead and disappeared, S = percentage surviving. If the onset of pyknosis is delayed, curve B moves to position C. Based on data for lymphocytes.

known directly. The best we can do is to make a similar count on a parallel batch of unirradiated cultures, express the experimental as a percentage of the control and assume that this is the "percentage survival." The assumption made, of course, is that two parallel batches of normal cultures contain the same total number of cells. How far this is likely to be true can be determined in preliminary experiments, and the error, which is the chief one of the method, can at any rate be assessed. This method is numerically less accurate than the dead cell counting method, but it does give the true percentage survival and this is the most valid measure of radiation effect. Experience has taught us that this is the better of the two methods; the gain in validity outweighs the slight loss in accuracy.

These various points may become clear if I now describe some studies on

the radiosensitivity of the lymphocytes in cultures of rat lymph nodes, in which each of these two methods has been employed.

The Radiosensitivity of Lymphocytes in Lymph Node Cultures, and the Effect of Oxygen

Counting pyknotic lymphocytes. This is old work (Trowell, 1952*b*, 1953*a*) in which an earlier pattern of culture apparatus and serum medium were used (Trowell, 1952*a*). The lymph nodes were the lumbar and sacral of 4-week-old rats. Later experiments showed that the sensitivity of these nodes is just the same in the new apparatus and in T8 medium, so these technical differences seem unimportant.

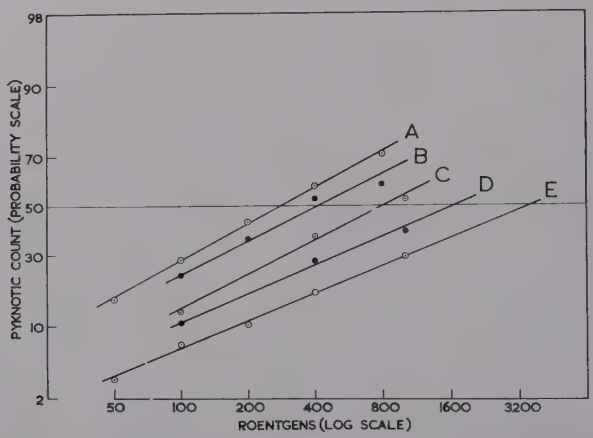


FIGURE 7. Lymph node cultures. The percentage of lymphocytes pyknotic 5 hours after exposure to various doses of X radiation. Irradiated in the following percentages of oxygen: A-100, B-60, C-21 (air), D-10.5, E-0 (nitrogen). Reproduced by permission of the *British Journal of Radiology* (Trowell, 1953*a*).

Five hours after irradiation each culture was reduced to a cell suspension from which a wet-fixed film preparation was made. The films were stained with Haemalum and the percentage of pyknotic (dead) lymphocytes was determined by counting. In each culture a total of 500 or more lymphocytes was counted, and the results from 12 cultures were averaged to give the figure for each radiation dose. The 5-hour end point, corresponding to T1 in FIGURE 6, was chosen because by then the radiation effect is about 60 per cent developed but most of the dead cells are still in a fairly early stage of pyknosis and easy to count. At later times the counts are progressively less accurate because some of the pyknotic nuclei break up into separate fragments. In any case there is no point in waiting longer than 5 hours because, as shown in FIGURE 6, the final percentage of cells killed can never be determined.

Curve A in FIGURE 7 gives the results obtained with cultures in 100 per cent oxygen, and shows that the probit of the effect was linearly related to the logarithm of the dose. This is the relationship usually found in the case of

drugs or toxins acting on a population of cells, and it simply represents the cumulative frequency distribution curve of the individual-cell sensitivities.

We were particularly interested in the protective effect of anoxia, which at that time had been found in various plant and insect material but had not been shown in normal mammalian cells. Curves B, C, D, and E are the results obtained in progressively lower concentrations of oxygen, E being in nitrogen. In each case the gas phase in the chamber was changed (from 100 per cent oxygen) 25 minutes before irradiation and changed back to 100 per cent oxygen immediately after irradiation. The doses that produce 50 per cent pyknosis

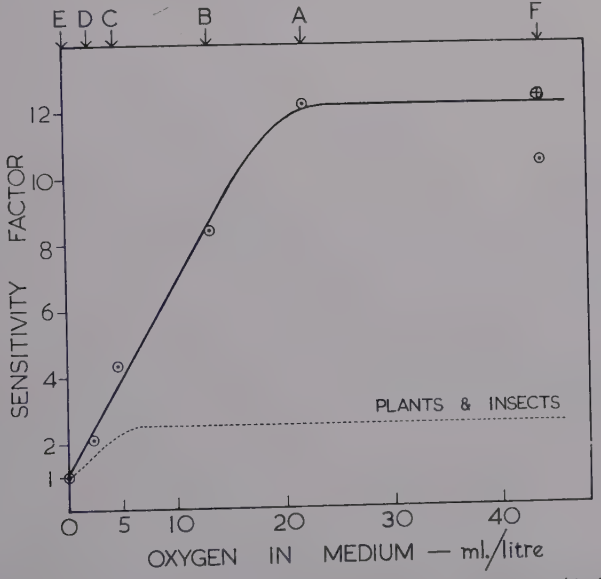


FIGURE 8. Lymph node cultures. The "oxygen sensitivity factor" (derived from FIGURE 7) plotted against the calculated concentration of dissolved oxygen in the culture medium. F = 2 atmospheres oxygen, ⊕ = point corrected for dose rate. Reproduced by permission of the *British Journal of Radiology* (Trowell, 1953*a*).

can be read off from FIGURE 7 and the ratio dose in nitrogen/dose in oxygen gives the "oxygen sensitivity factor" in each case. In FIGURE 8 the oxygen sensitivity factor is plotted against concentration of dissolved oxygen in the medium, and an experiment performed with oxygen at 2 atmospheres pressure is also included (F). It is seen that "maximum oxygen sensitivity factor" is 12, which means that by increasing the oxygen concentration from zero the radiosensitivity of these lymphocytes can be increased 12-fold.

This was a very surprising result because in all the other biological systems investigated the maximum factor had turned out to be between 2.5 and 3.5. This is an important question to which I shall return in a moment.

It was shown that decreasing the *p*H of the medium, just before irradiation, from *p*H 7.3 to 6.3 and 6.0 appeared to decrease radiosensitivity, whereas increasing it from *p*H 7.3 to 8.3 had no effect. These results are shown in

FIGURE 9, where curve K was obtained at *p*H 7.3, P at *p*H 6.3, N at *p*H 6.0, and A at 8.3. It was also found that addition of lactate (0.02 *M*) decreased radiosensitivity (curve R in FIGURE 9). Variation of glucose concentration within the range 0.1 to 0.6 per cent and variation of CO_2 in the gas phase within the range 0 to 7 per cent had no effect; neither did changing the medium from serum to Tyrode solution. It was also interesting to find that the radiosensitivities of lymph nodes taken from rats reared and housed in two separate

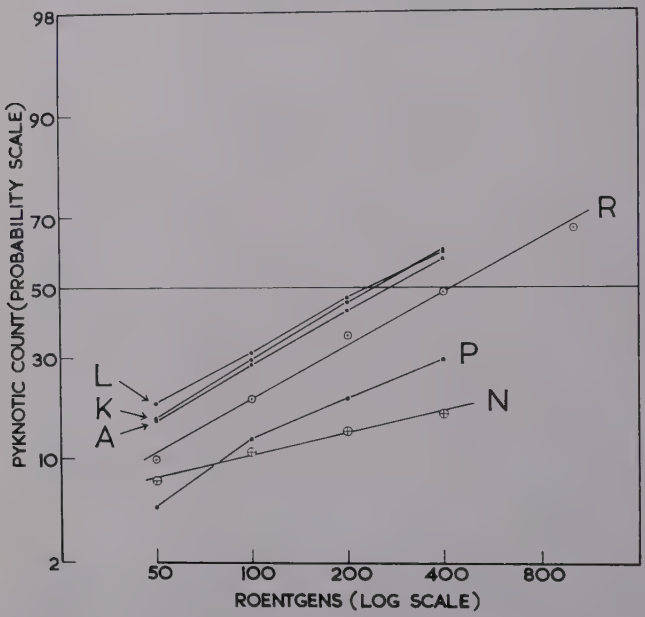


FIGURE 9. Lymph node cultures. The percentage of lymphocytes pyknotic 5 hours after exposure to various doses of X radiation in 100 per cent oxygen. A = lymph nodes from Harwell rats, irradiated at *p*H 8.3, K = the same, irradiated at *p*H 7.3, L = lymph nodes from a different rat colony (A.R.C. Field Station, Compton, Berkshire), irradiated at *p*H 7.3. In P, N, and R, additions were made to the medium just before irradiation, thus: P = 0.017 *M* HCl (*p*H 6.3), N = 0.025 *M* HCl (*p*H 6.0), R = 0.02 *M* lactate. Reproduced by permission of the *British Journal of Radiology* (Trowell, 1953*a*).

laboratories and fed different diets were almost identical (curves K and L, FIGURE 9).

Soon after this work was published I began to suspect that the apparent changes in radiosensitivity produced by oxygen and *p*H might be partly fallacious. I reasoned that reducing the oxygen concentration or the *p*H might simply delay the onset of visible pyknosis in cells already killed by radiation, without necessarily altering the total number killed. In this case the pyknosis/time curve shown in FIGURE 6 would be displaced to the right and, instead of curve B, we should record curve C. Reading at T_1, this would register as a fall in radiosensitivity, additional to any genuine change which might occur. It is now generally agreed that nuclear pyknosis is a post-mortem phenomenon resulting from the action of autolytic enzymes on dead cells, so it seemed not

unlikely that the activity of these enzymes should be influenced by *p*H and other factors in this way. However this was just a hypothesis, and I knew of no experimental work on this aspect of pathology. Recently, however, Ruth Moore (1961) has found that injection of Nembutal into animals just after irradiation delays the development of pyknosis by about one hour. So it seems that changes of this sort can occur. If so, then addition of any protecting or sensitizing chemical might produce them and apparent changes in radiosensitivity found by pyknotic counting may always be fallacious. This probably applies to all organs, not just lymph nodes, and it may also apply when other criteria of cell death such as eosin stainability are used.

It therefore became rather urgent to find some method of measurement free from this objection and then to repeat the experiments and see if identical sensitivity changes could be observed. The method required would have to involve live cell counting and give the percentage survival because this would not be affected by small changes in the speed of pyknosis. The method eventually devised will now be described.

Counting surviving lymphocytes. For each determination it was necessary to set up two parallel chambers each containing 20 lymph node cultures, using the method described above under *Organ-Culture Method*, and it was essential that the two sets should be very closely matched in both size and origin. The mesenteric nodes of 4-week-old rats were used because they are of convenient shape and size, and the number of lymphocytes per unit volume is more constant than in other nodes. These nodes collectively represent the pancreas of Aselli, which can be regarded as a cord of lymphoid tissue about 2 mm. wide, constricted across at irregular intervals. The individual nodes are thus very variable in length but rather uniform in width, as shown in FIGURE 10. By careful transections with cataract knives it is possible to produce a large number of cultures of fairly uniform size (FIGURE 10). The nodes from one animal are sufficient to provide 20 cultures. These 20 cultures are planted alternately into the 2 chambers, keeping them strictly in the order in which they were prepared as indicated by the numbering in FIGURE 10. From a second animal 20 additional cultures are prepared and planted in exactly the same way. This system ensures that if a node is cut into 2 pieces 1 goes into each chamber; if it is cut into several pieces approximately one half of them go into each chamber, and the material from each animal is uniformly distributed between the 2 chambers.

On the following day one chamber is irradiated and the other "mock irradiated," which means it is subjected to exactly the same procedures, including alteration of the medium or gas phase if this is done, but the X-ray shutter is not opened. Exactly 24 hours after irradiation or mock irradiation the total number of lymphocytes in each chamber is determined as follows.

The 20 cultures are transferred to an embryo dish containing 2 ml. of 0.1 *M* citric acid. This fixes the cells at once and then slowly dissolves the cytoplasm so that ultimately a suspension of free cell nuclei is obtained. It is interesting to recall that this acid method for isolating cell nuclei was described by Robin in 1850, about the same time as the lymphocyte was first recognized. After an hour's fixation the cultures are cut up and teased to bits with cataract

knives. The material is then quantitatively transferred to a stoppered test tube and made up to 10 ml. with citric acid. Then 1 ml. of 0.1 per cent gentian violet and 3 glass beads are added and the tube is well shaken. After high doses of radiation only one half of this volume of suspension fluid is used (5.5 ml.). After standing overnight the tube is again shaken and samples are counted in a Fuchs-Rosenthal chamber, counting healthy lymphocyte nuclei only. From this count the total number of healthy lymphocytes in the 20 cultures is obtained.

The total number of lymphocytes in the irradiated cultures as a percentage of the total in the control is taken to be the percentage survival. This of

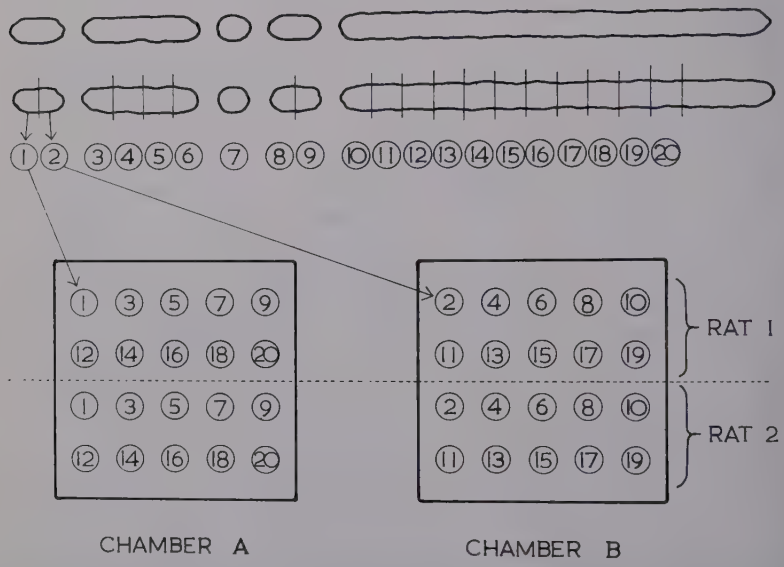


FIGURE 10. Scheme for the preparation of 40 cultures from mesenteric lymph nodes and their distribution into two matched chambers, A and B. Top row shows the chain of mesenteric lymph nodes from one rat, which collectively represent the "pancreas of Aselli." Second row shows how these are cut transversely. Third row shows the cultures so produced and their allocation to the two chambers.

course involves the assumption that the total number of lymphocytes in the 2 chambers was the same to start with and that, in the absence of irradiation, it would still have been the same after the 2 days *in vitro*. Naturally this ideal cannot be fully realized, but it was established by experiment that the total lymphocytes in 2 parallel chambers, both initially and after 2 days *in vitro*, did not differ by more than 10 per cent; usually the difference was less than 7 per cent. The total number was about 30 million.

Our first aim was to establish the dose-effect curves in oxygen, in air, and in nitrogen (plus 5 per cent CO_2 in each case) and the results thus far obtained are shown in FIGURE 11. Each point is the average of 2 to 4 separate determinations. Since the true percentage survival was measured (subject to technical error), the logarithm of the percentage surviving has been plotted

against dose in the conventional way and a linear relation is to be expected. The points do appear to fall approximately on straight lines, and the extrapolated lines pass through or close to zero, which shows that there is no dose threshold or, at best, a very small one. The main interest however is the oxygen effect. The 50 per cent survival dose was 105 r in oxygen and 280 r in nitrogen, so the "maximum oxygen sensitivity factor" is now found to be 2.7. This agrees well with the values (2.5 to 3.5) found in almost all other biological materials, and, more specifically, with the values of 2.5 for thymus lymphocyte *in vitro* (Patt, 1955), 3.55 for rat-lymph nodes *in vivo* (Moore, 1961), and 2.4 to 2.8 for mouse thymus *in vivo* (Wright and Batchelor, 1959). I feel fairly confident that this is the right answer and that the value of 12 ound in our earlier work by pyknotic counting was fallacious, probably be-

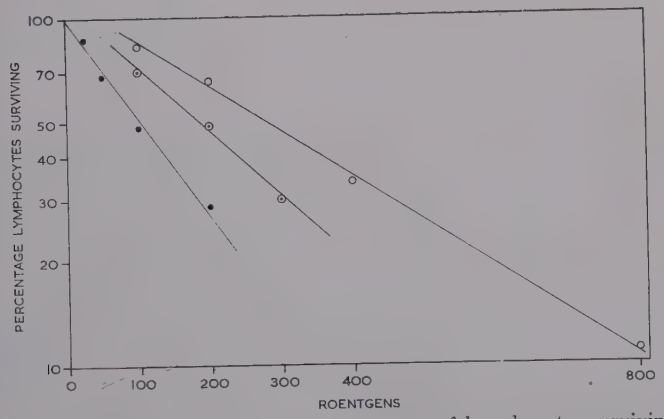


FIGURE 11. Lymph node cultures. The percentage of lymphocytes surviving 24 hours after exposure to various doses of X radiation. Irradiated in oxygen (●), air (⊙), and nitrogen (○), plus 5 per cent of CO_2 in each case.

cause prior exposure of the cells to anoxia delays the subsequent onset of radiation-induced pyknosis.

I have dealt at some length with the technical aspects of this work on the oxygen effect because it does bring out the chief point I am trying to make in this paper: that accurate measurement of the radiation effect is difficult, that different end points may appear to yield different results, and that the results should be interpreted only in the strict light of the end point chosen; wider interpretation may lead to fallacious conclusions.

The results obtained by counting surviving lymphocytes in this way are numerically less accurate than those obtained by pyknotic counting, but they do provide the sort of information we desire. We shall therefore continue to use this method and perhaps improve its accuracy by having more cultures in the chamber. There should be no difficulty in scaling up the horizontal dimensions of the whole culture apparatus so as to accommodate 100 cultures. It is hoped to study the effects of pH and other physiological variables and then go on to the various protective and sensitizing chemicals. In connection with protective substances it may be remarked that a direct action on the cells

can never be conclusively demonstrated in whole-animal experiments because of the possibility of a concomitant fall in tissue oxygen tension. However with this organ culture system we can always irradiate in nitrogen and so exclude possible changes in oxygen concentration. This seems to me about the only way in which fundamental knowledge of the effects of protective or sensitizing substances on normal mammalian cells can at present be obtained.

Differential Cell Counting

Brief mention may now be made of another method of cell counting that can be used in a few special instances. In any organ it is usually the case that one type of cell is radiosensitive (S) while some other type is resistant (R), within the dose range used. If the numerical ratio S/R could be determined in both control and irradiated specimens, the percentage survival of S could be computed. Thus if S/R was found to be 9 in the controls and 3 in the irradiated, the deduction is that two thirds of the S population had disappeared and the survival was 33 per cent.

The required differential cell counting is done on histological sections, counting the cell nuclei. An eyepiece graticule in the shape of a straight line or a ring (depending on the material) is used, and the numbers of the two sorts of nuclei (S and R) that are touched or intersected by the line are counted. The theory and details of the method, which can also give the absolute number of cells in unit volume, have been described by Trowell and Westgarth (1959). The point to make here is that it can only be used on organs in which either (1) the distribution of S and R is reasonably homogenous (for example, thymus), or (2) S and R are arranged in separate parallel layers (for example, retina). In the former case a ring graticule is used, in the latter a line graticule orientated at right angles to the layers.

We have used this method on the thymus, where S are the lymphocytes and R the reticulum cells, but so far only *in vivo*. Satisfactory culture of the thymus still eludes us. However the beauty of this method is that cortex and medulla can be counted separately, and, using rat thymus *in vivo*, we were able to show that the lymphocytes in the cortex are four times more radiosensitive than those in the medulla (Trowell, 1961*b*).

The method can be used for the retina, where S are the visual cells (rods and cones) and R the bipolar cells, and some results are described below. In the examples cited, the live cells remaining after irradiation are counted and a true percentage survival is obtained.

The Radiosensitivity of Visual Cells in Retinal Cultures

Lucas and Trowell (1958) reported the satisfactory survival of the isolated retinae of mouse and rat in organ culture. The method used was that described above under *Organ-Culture Method* except that oxygen was found to be toxic, so air was used instead. Since then my colleague, D. R. Lucas, has found that the fully-differentiated retina of 6- to 11-day-old guinea pigs is more satisfactory for radiation studies *in vitro*, and he has kindly allowed me to report his preliminary results. Each whole retina was cut into 4 pieces, and 4 pieces were cultured in each chamber in air plus 5 per cent CO_2. The cultures

were fixed and sectioned 18 hours after irradiation, and control cultures were carried through at the same time. The ratio of visual to bipolar cell nuclei in the sections was measured by the line-counting method described above under *Differential Cell Counting*, and the percentage survival of the visual cells was deduced. The results are shown in FIGURE 12, where each point is the average of only 2 cultures. These are preliminary results, but they suffice to show a striking contrast to the lymphocyte. The 50 per cent survival dose for these guinea pig visual cells is about 2250 r, compared with 190 r for the rat lymphocytes (in air). Even so, visual cells come within the category ordinarily re-

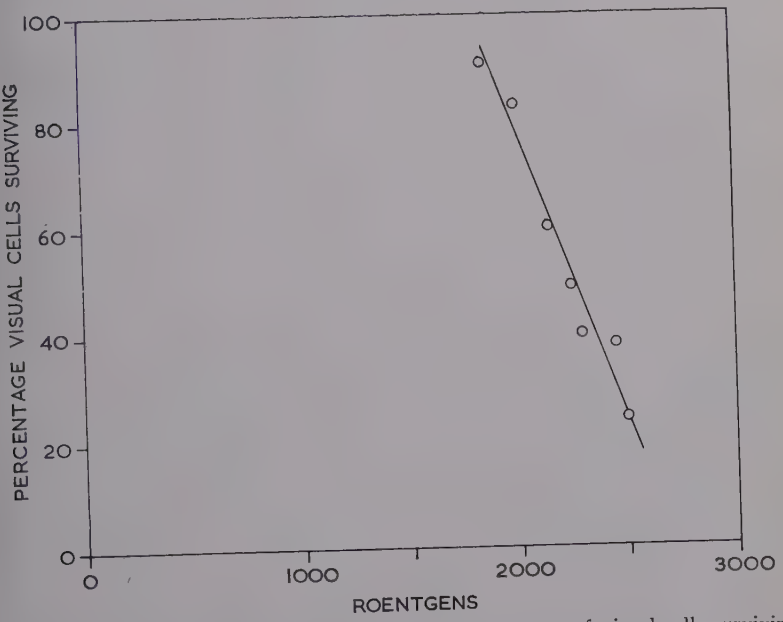


FIGURE 12. Retina cultures (guinea pig). The percentage of visual cells surviving 18 hours after exposure to various doses of X radiation in air plus 5 per cent of CO_2. Experiments of D. R. Lucas (unpublished).

garded as radiosensitive. It is also interesting to see that the visual cells have a very high dose threshold, probably about 1500 r, whereas the lymphocytes had virtually no threshold. The sensitivity range of the individual visual cells seems to be remarkably small, again in contrast to lymphocytes.

Concluding Remarks

All the methods I have described for measuring the radiation effect are morphological. They involve accurate discrimination of cell nuclei and laborious counting. Perhaps chemical methods could be used, measuring either the loss of some cell component such as DNA or potassium, or the reduction of some metabolic parameter such as consumption of oxygen or glucose. We have tried a few of these chemical methods but thus far have not found one that can approach the accuracy of cell counting. An inherent advantage of cell

counting in these organ cultures is of course that attention is confined to the radiosensitive cells; the others can be entirely disregarded unless required for computing a ratio. With chemical methods this is not possible; one has to deal with the whole culture and, if the radiosensitive fraction is small, the chemical changes will be small even when all the sensitive cells are killed.

There is one other radiobiological application of this organ-culture method not yet mentioned. This is to study the radiotoxic (cell killing) effect of various radioisotopes on specific cell types. Very little is known about this because in whole-animal experiments the exact concentration of isotope to which the cells are exposed is not known and, in any case, this concentration cannot be held steady. By simply adding isotope to the culture medium it should be possible to measure the effects on specific cell types of known concentrations maintained for known lengths of time. Again there seems to be no other way in which this important information can be obtained. I have previously measured the toxic effects of cortisone (1953b), barbiturates (1958) and mitotic poisons (1960) on lymphocytes in lymph-node cultures, and it should be possible to study radioisotopes in the same way.

The extension of this work to other organs is at present somewhat limited by technical difficulties. In particular, the *in vitro* survival of bone marrow, thymus, and brain is unsatisfactory and there is no immediate hope of improvement. However, there are many other organs that survive quite well (Trowell, 1959), although in these it may be found that the present cell-counting methods are inadequate. I think that further progress in this field of radiobiology will depend very much on the development of new and better methods for measuring radiation effects and perhaps on discovering new effects to measure. It must be remembered, however, that none of these effects is likely to be radiation-specific; these effects are the common coin of pathology. We must look for and contribute to the development of "quantitative histopathology," a discipline that accepts the concepts of classical descriptive pathology and seeks to delineate them by precise measurement.

Acknowledgments

Work of this sort depends for its success, if any, on a sustained attention to technical detail. I therefore pay a warm tribute to my technical assistants, W. R. Lush, E. Peakman, M. Tebbutt, and C. Ash, for their painstaking work in organ culture and cell counting, and to M. J. Corp for similar care with the irradiations and dosimetry. I also thank my colleague, D. R. Lucas, for his general collaboration and for permission to report some of his unpublished work.

References

Lucas, D. R. & O. A. Trowell. 1958. *In vitro* culture of the eye and the retina of the mouse and rat. J. Embryol. Exptl. Morphol. **6:** 178–182.

Moore, R. 1961. The oxygen-effect factor for rat popliteal lymph nodes irradiated *in vivo*. Radiation Research. **114:** 296–307.

Patt, H. M. 1955. Factors in the radiosensitivity of mammalian cells. Ann. N. Y. Acad. Sci. **59**(4): 649–664.

Trowell, O. A. 1952*a*. The culture of lymph nodes *in vitro*. Exptl. Cell Research. **3:** 79–107.

Trowell, O. A. 1952*b*. The sensitivity of lymphocytes to ionising radiation. J. Pathol. Bacteriol. **64:** 687–704.
Trowell, O. A. 1953*a*. The effect of environmental factors on the radiosensitivity of lymph nodes cultured *in vitro*. Brit. J. Radiol. **26:** 302–309.
Trowell, O. A. 1953*b*. The action of cortisone on lymphocytes *in vitro*. J. Physiol. (London). **119:** 274–285.
Trowell, O. A. 1958. The cytocidal action of barbiturates on lymphocytes *in vitro*. Biochem. Pharmacol. **1:** 288–295.
Trowell, O. A. 1959. The culture of mature organs in a synthetic medium. Exptl. Cell Research. **16:** 118–147.
Trowell, O. A. 1960. The cytocidal action of mitotic poisons on lymphocytes *in vitro*. Biochem. Pharmacol. **5:** 53–63.
Trowell, O. A. 1961*a*. Problems in the maintenance of mature organs *in vitro*. Colloques internationaux du Centre National de la Recherche Scientifique, No. 101. La culture organotypique. Editions du C. N. R. S. Paris, France.
Trowell, O. A. 1961*b*. The radiosensitivities of the cortical and the medullary lymphocytes in the thymus. Intern. J. Radiation Biol. In press.
Trowell, O. A. & D. R. Westgarth. 1959. Anat. Record. **134:** 463–471.
Wright, E. A. & A. L. Batchelor. 1959. The change in the radiosensitivity of the intact mouse thymus produced by breathing nitrogen. Brit. J. Radiol. **32:** 168–173.

THE EFFECT OF IONIZING RADIATIONS ON MOUSE EMBRYONIC LUNGS DEVELOPING *IN VITRO*

Elio Borghese

Divisione Biologica, Comitato Nazionale per l'Energia Nucleare, Frascati, Italy

A part of the research now in progress at the Embryological Laboratory of the Biology Division of Comitato Nazionale per l'Energia Nucleare at Frascati, near Rome, belongs to a subject involved in the problems studied by the present symposium: the action of ionizing radiation on embryonic organs differentiating *in vitro*.

It has been known for a long time that when ionizing radiation is applied to pregnant females malformed embryos are produced. The publications on this subject are very numerous; however, on this occasion I refer chiefly to the work by L. B. Russell (1950, 1954, 1956) at Oak Ridge, which is closely related to the present research. Russell has shown, through a systematic investigation on mouse embryos, that the effects of radiation can be grouped, according to stage of development, in three periods: preimplantation, major organogenesis, fetus. In addition, her work has confirmed that radiation is very variable in its effect when given to a great number of individuals, so that the results of such research cannot be expressed except in terms of statistical frequency.

The developmental stage being studied in our laboratory corresponds to the second stage described by Russell, namely the period of major organogenesis: the developmental time in which some organs begin to have a well defined shape, may be isolated easily from their surroundings, and are capable of going on to grow and differentiate *in vitro* and, in some days of culture, to exhibit rather complex developmental processes.

The first research, which has gone far enough to allow us to present here some preliminary results, concerns the development of mouse lung. It is being done by T. Alescio, an embryologist, and M. Ladu, a physicist.

In a previous work Alescio (1960) had shown that the lung rudiment of mouse, isolated at the 12th day of gestation, when the bronchial tree consists of primary bronchi just beginning to bud off secondary ones, may be cultivated as long as 6 days in a watch glass according to Fell and Robison's technique (1929). In these conditions it becomes a complicated bronchial tree with dozens of branches: the histological differentiation of the epithelium follows a pattern very similar to the normal; bronchial folds are produced and the ends of the smallest twigs develop irregular, mesenchymelike coverings.

The same organ is now being subjected to the action of different doses of gamma radiations from a Co^{60} source of the original activity of 500 mC., in order to see how it behaves when the action of the whole embryonic body and of the mother are removed.

The irradiation technique was as follows. Mouse embryos of 2 different strains, a Swiss albino and a strictly inbred C 57 black, were used. No difference was noted between the 2 strains in the results of the research. At the 12th day of gestation, all the lungs of the litters were removed and divided into two batches, which were put in separate test tubes containing a mixture

of Tyrode (2 parts), horse serum (1 part), and chick embryo extract (1 part). After several trials, this fluid was proved to be almost harmless for the period of about one hour required for the steps of the irradiation. One of the tubes was kept as a control; the other was inserted in the apparatus shown in FIGURE 1, a Lucite block containing a row of holes at the measured distance of 5 mm. The tube containing the explants was put into one hole and the source into another. By means of combinations of distance and of irradiation time the administration of any required dose of irradiation was possible. The results

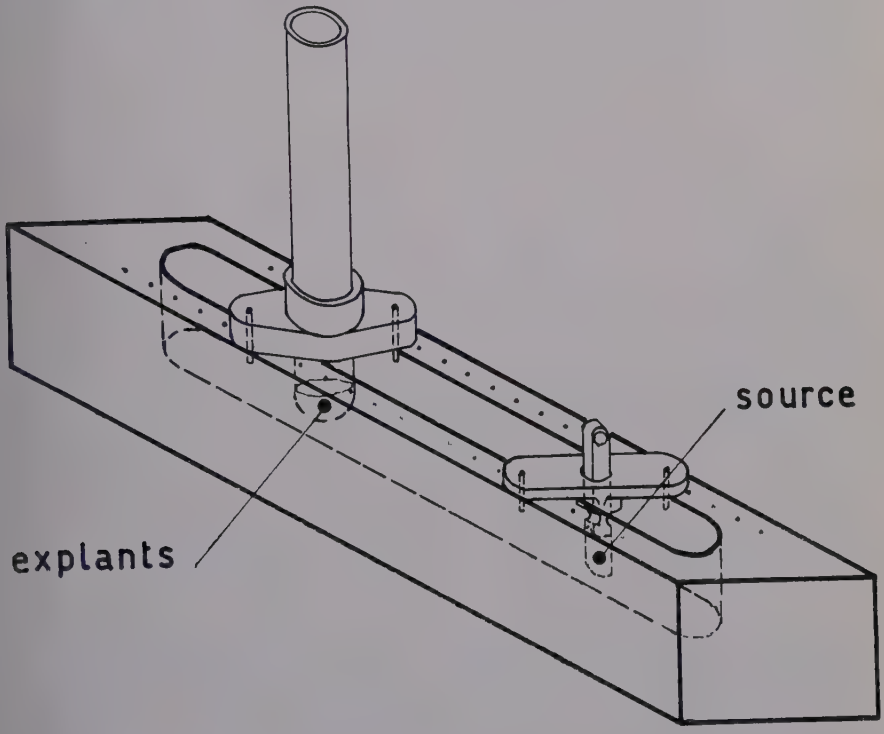


FIGURE 1. Lucite block used for irradiation.

to be presented here refer only to the experimental setting shown in FIGURE 2, where the distance between source and explants is 15 mm. Taking into account the decay curve of the source, which gave 465 mC. as its actual activity, and with an irradiation time of 18 min. at this distance, the calculated dose is 818 r, if the source is considered pointlike ("puntiform"), which is sufficiently approximate to its real shape. This dose is surely high and damaging enough to obtain first information on the histological injury produced.

After irradiation, the explants are washed several times in Tyrode's solution and laid on clots of chick plasma and embryo extract, some according to hanging drop technique, but most according to watch-glass technique. At the same time control cultures were made, using the rudiments that were kept

apart in the second test tube and had remained for an equal length of time in the solution. Both control and irradiated cultures were examined in the living state every few hours on the first day and later every day: camera-lucida drawings and motion pictures were made. The cultures were then fixed at different times of cultivation: 6, 18, and 24 hours, and 2, 3, and 4 days; then sectioned and stained.

In another experiment, some pregnant female mice on the 12th day of gestation were irradiated on the abdomen with an approximately equal dose of gamma rays from Co^{60}; they were killed on the 16th day, and the embryos were removed and studied.

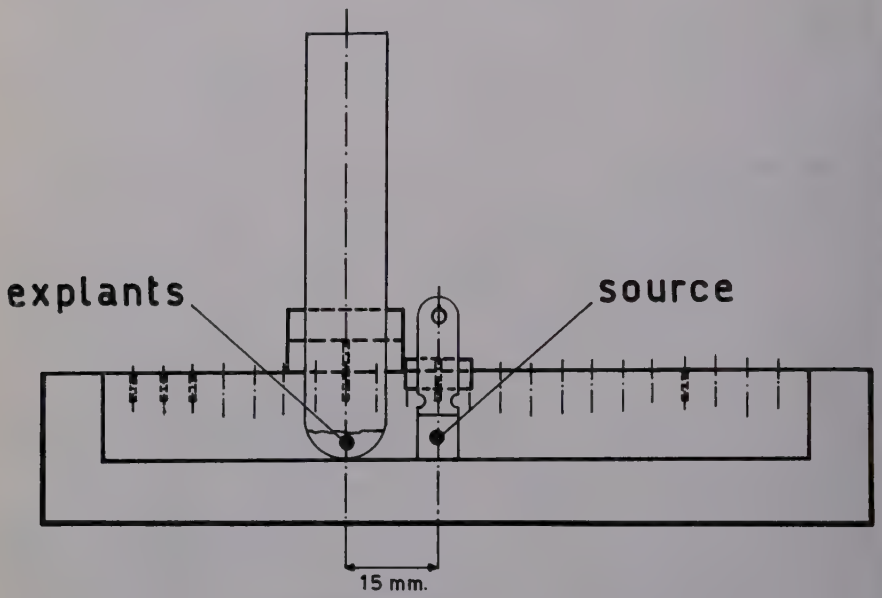


FIGURE 2. Actual setting of the experiment. The distance between the Co^{60} source and the explants is 15 mm.

The chief results thus far are summarized here.

(1) All irradiated cultures, observed at a given time from the beginning of cultivation, were injured more or less in the same way: no variability was found among them comparable to what was already known and confirmed by the control research when the radiation was administered to embryos growing *in utero*. In other words, under the conditions of the present experiments, carried out on isolated organs under a rather heavy dose, all explanted lungs were damaged uniformly, so that a simplification of experimental conditions was reached. Further research is required to see whether this result will be repeated under lower doses.

(2) When a dose sufficient to cause large malformations of certain nonpulmonary organs was administered to pregnant females, it did not cause in the specimens studied any alteration of the lungs. From the general literature on the effect of radiation, it is known that the lung is a rather resistant organ.

In the isolated lungs this resistance evidently has been overcome, for irradiation of the removed lungs *in vitro* did damage these organs.

(3) The cultured lungs, both control and irradiated, were studied in 2 ways: by means of direct observation in the living state accompanied by camera-lucida drawings and motion pictures, and by observation in sections of fixed and stained cultures. FIGURE 3 shows a nonirradiated control culture drawn in successive stages until the 30th hour after explantation: one sees the branching process of the bronchial tree proceeding for some time, and also a certain degree of growth that may be due more to water absorption than to real increase of cellular volume. After the last stage represented in the drawing, the explanted lung continues to branch for 2 or 3 days, but the superimposition of the twigs becomes so complicated that no drawing can satisfactorily portray

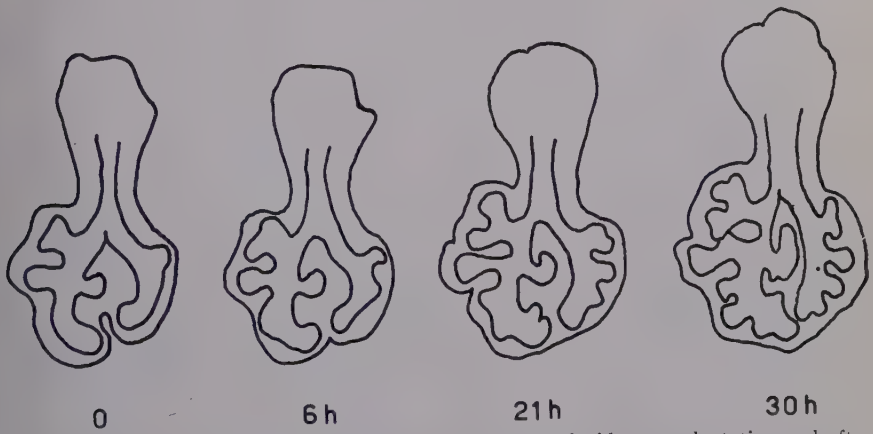


FIGURE 3. Nonirradiated culture drawn under camera lucida at explantation and after 6, 21, and 30 hours. The branching continues for the next days, but superimposition of the branches prevents drawing the outlines.

it; only sections are able to give an idea of the extreme intricacy of the bronchial tree. The behavior of an irradiated culture is quite different, as shown in FIGURE 4. A few minutes after irradiation, the explant begins to become opaque at the middle of the rudiment, the place where the trachea joins the bronchi. The opacity spreads out very rapidly and remains so for about two days. However at the end of the second day some clearing begins to take place in the middle of the culture; in many cases the material in the lumina responsible for the darkness of the explant appears to be expelled through the laryngeal orifice. The trachea and primary bronchi become visible again and, at the fourth day of culture, the whole explant has recovered sufficient transparency to make it possible for the observer to ascertain that no substantial branching has taken place. The explant is strongly diminished in size in comparison with the original one. A schematic comparison of the two behaviors is presented in FIGURE 5.

The events thus far discussed can be shown also in a time-lapse motion picture that shows the behavior of irradiated and nonirradiated cultures. More-

over, the film reveals a pulsating movement of the whole lung that is very slow in the controls and faster and stronger in the irradiated lung. The meaning of this pulsation, which can be detected only by means of accelerated cinematography and is described in C. M. Pomerat's study of oligodendroglia cells (1951) is not known.

(4) The sections made at different stages of the cultures help to explain the opacity of the irradiated cultures. They show a rather unexpected differential sensitivity of epithelium and mesenchyme, depending on the age of the culture.

When the culture is fixed after six hours of cultivation, an extensive degeneration of the epithelium is seen: the dead cells detach in great numbers and pass into the lumina of the bronchi, which become completely filled. Spherules

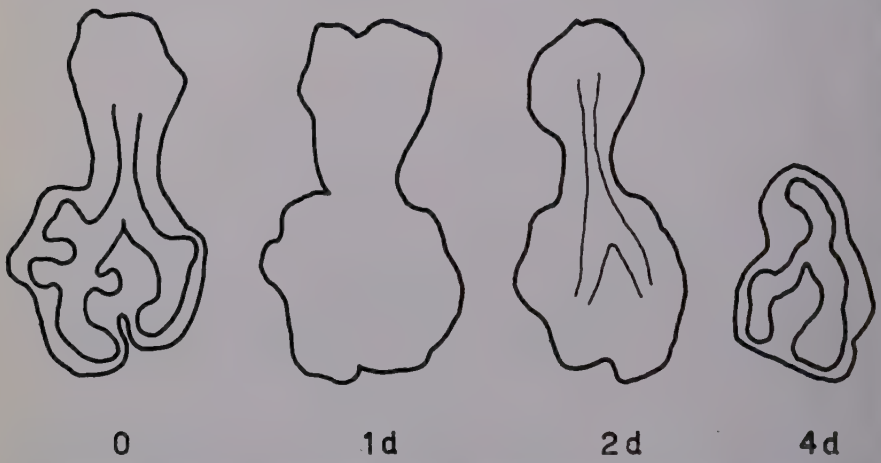


FIGURE 4. Irradiated culture drawn under camera lucida at explantation and after 1, 2 and 4 days. In a few hours the culture becomes so dark that portrayal of it by drawing becomes impossible: it clears up later and shows that no branching has occurred.

of strongly stained material, presumably nuclear, are seen both in the epithelium and inside the cavities, together with a great deal of other cellular debris. Mitoses, very frequent in control cultures, are missing in the irradiated ones. No injury of the mesenchymal cells was detected at this stage. This picture lasts about 18 hours, during which time the bronchial tree does not branch; instead, it shows about the same outlook as that at explantation time. The most obvious interpretation of the lack of branching may be found in the strong epithelial degeneration: even if an inductive action of the mesenchyme were present, according to C. Grobstein's research (1955), the actual formation of new buds would be due to epithelial movements that would not be possible unless all cells concerned were in good health.

At two days of culture the content of the bronchi has changed: cellular debris is no longer found, but only a thin mesh, perhaps a clotted fluid. The epithelium is nearly normal and is provided with a substantial number of mitoses; some epithelial folds begin to appear. The mesenchyme, which was almost unimpaired during the first days, shows much pyknotic degeneration.

In so far as may be judged at the present time, two successive phases take place, apparently as the effect of radiation. First, the epithelium is selectively injured; it stops growing and branching; many of its cells degenerate and fall

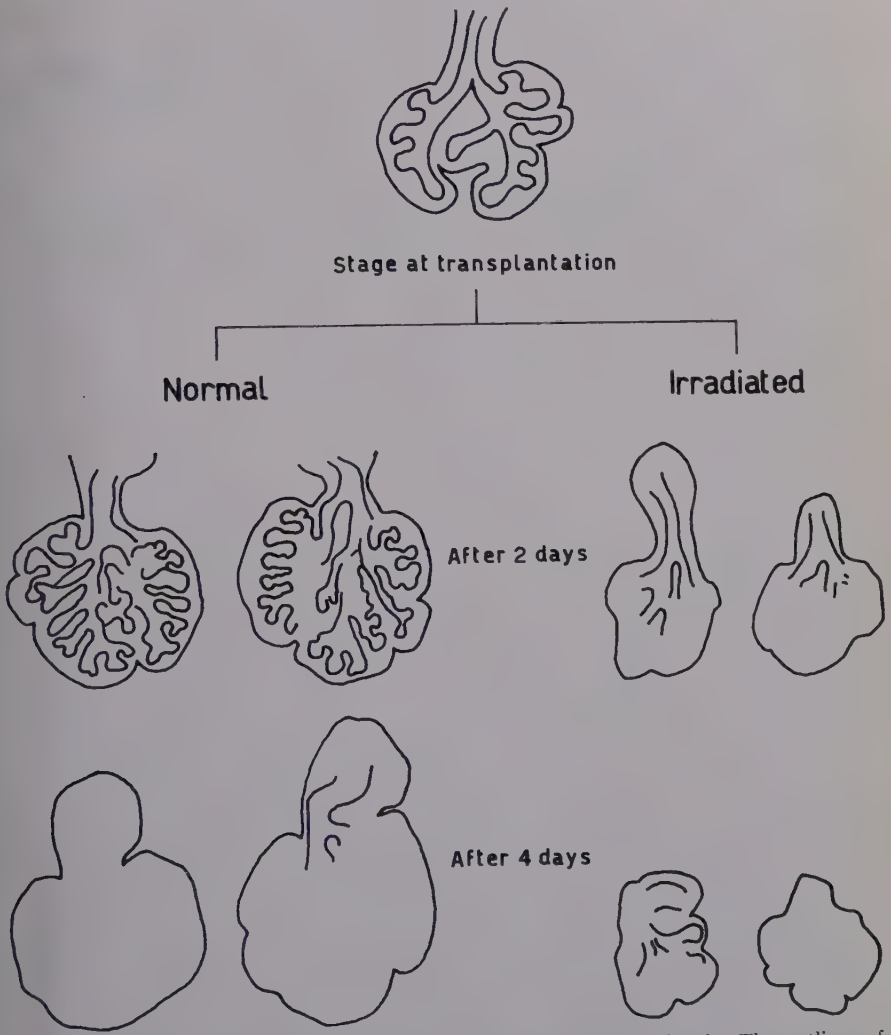


FIGURE 5. Comparison of the cultures, irradiated and nonirradiated. The outlines of all cultures were not drawn at the 4-day stage. The general contour shows the difference in size, including growth in the nonirradiated lung and strong contraction in the irradiated.

into the lumen. Second, the epithelium begins to regenerate, as proved by the numerous mitoses, and does not lose any more cells. The mesenchyme is in a state of diffuse pyknosis. This is the typical picture of the irradiated cultures at the 4th day.

In spite of the comparatively good condition of the epithelium, which ap-

pears to have regenerated in substantial degree, the ramification activity, lost at the beginning, does not reappear.

References

Alescio, T. 1960. Osservazioni su culture organotipiche di polmone embrionale di topo. Arch. ital. Anat. Embriol. **65:** 323–363.

Fell, H. B. & R. Robison. 1929. The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated *in vitro*. Biochem. J. **23:** 767–784.

Grobstein, C. 1955. Tissue interaction in the morphogenesis of mouse embryonic rudiments *in vitro*. *In* D. Rudnick, Aspects of synthesis and order in growth. : 233–256. Princeton Univ. Press. Princeton, N. J.

Pomerat, C. M. 1951. Pulsatile activity of cells from the human brain in tissue culture. J. Nervous Mental Disease. **114:** 430–440.

Russell, L. B. 1954. The effects of radiation on mammalian prenatal development. *In* Radiation Biology. A. Hollaender, Ed., McGraw-Hill, New York, N.Y. **1**(2): 861–918.

Russell, L. B. 1956. X-ray induced developmental abnormalities in the mouse and their use in the analysis of embryological patterns. I. External and gross visceral changes. J. Exptl. Zool. **114:** 545–602.

Russell, L. B. 1956. X ray induced developmental abnormalities in the mouse and their use in the analysis of embryological patterns. II. Abnormalities of the vertebral column and thorax. J. Exptl. Zool. **131:** 329–396.

THE EFFECT OF X RAYS ON CELLULAR DIFFERENTIATION IN ORGAN CULTURE

Ilse Lasnitzki*

Strangeways Research Laboratory, Cambridge, England

The application of the tissue-culture method to the study of radiobiology goes back nearly 50 years. In 1914, Mottram[1] described diminution of cell division in cultures of sarcoma and carcinoma cells exposed to gamma rays from radium. The study was put on a more quantitative basis by Strangeways;[2] it was extended by many other workers, among them Spear,[3] Paterson,[4] and myself[5-7] who, by quantitative analysis, investigated the effects of radiation and of the variation of physical factors on mitosis, cell death, and mitotic recovery in culture. The same criteria were used in a comparison of the radiation effect on tumor cells in culture and in the animal.[8]

This paper is concerned with another, less well-known effect of radiation, namely that on differentiation. In contrast to cell cultures most normal tissues in the organism consist of differentiated, that is, specialized cells and "reserve" cells that are capable of mitosis and replace worn-out specialized elements. In malignant tumors the balance of division and differentiation is disturbed in favor of division, but in many tumors a certain proportion of cells still proceed to differentiation and thus drop out of the mitotic race.

In his systematic study on the effects of radiation on human malignant tumors, Glücksmann[9-13] has shown that radiation increases both the number of differentiated cells and their degree of differentiation, and that this effect can be an important factor in the sterilization of certain tumors, particularly epidermoid carcinomas. His results have since been confirmed by many workers.

An increase in differentiation in response to radiation has been obtained in normal tissues also; thus in the intestine radiation promotes the maturation of stem cells to goblet cells.[10,11]

In both tumors and intestine the specialization of the cells follows the pattern laid down for the normal prototype, that is, the change is of a quantitative nature and probably secondary to an arrest of cell division. In other tissues, however, radiation was found to induce a qualitative change of differentiation. Examples of this are the squamous metaplasia of the mucous glands of the oral cavity after radiation,[12,13] and the alteration from a squamous toward a mucoid type of epithelium in the rat vagina exposed to X rays.[14]

It is not clear whether this qualitative change in differentiation is linked to mitotic inhibition or due to an effect on the cytoplasm. Endocrine factors may also play a role. The vaginal epithelium of rodents changes during the estrous cycle from a secretory to a squamous keratinizing epithelium, and it is possible that the alteration of differentiation *in vivo* might be caused by an effect of radiation on the endocrines that, in their turn, alter the balance of sex hormones in the animal.

This capacity for modulation[15] makes the vagina a suitable tissue in which

* Sir Halley Stewart Fellow.

to study the effects of radiation on differentiation in organ culture. By this means it should be possible to determine whether differentiation of the vaginal epithelium can be affected by radiation in the absence of sex hormones, and also whether this effect is altered by the addition of known concentrations of estrogenic hormones to the culture medium. The environment of the cultures may also influence the radiation effect; natural medium for instance contains small unknown quantities of hormones and vitamins that can be excluded from chemically defined medium, and it was thought of interest to compare the effects of radiation under these two conditions.

Experimental Methods

The vaginal epithelium was obtained from two-week-old mice of a laboratory inbred strain at a stage when the vaginal tubes were still closed. The organs were excised from the peritoneal cavity, opened, and divided into four parts, of which one served as control and the other three as experimental cultures.

Two variations of the orthodox watchglass technique by Fell and Robison[16] were used. For growth in natural medium the fragments were placed on strips of rayon acetate[17] that rested on a plasma clot; for growth in the defined medium the cultures were arranged on squares of lenspaper[18] that floated on the defined medium. The plasma clot consisted of a mixture of chick plasma, horse serum, and chick-embryo extract in a proportion of 2:1:1. The defined medium was a modification of the Healy and Parker medium No. 858.[19] The hormone was added to the medium in the form of estrone* in a concentration of 2 μg./ml.

Two sets of cultures grown in natural and defined medium and two others grown with addition of estrone were exposed to X rays 1 day after explantation. The X ray source operated at 200 kv at 10 mAmp. with a filtration of 0.5 mm. Cu and 1 mm. Al. A dose of 300 r U. was given at a dose rate of 42.7 r/min. at a focal distance of 50 cm.

After 3-days growth, the cultures were fixed in 3 per cent acetic Zenker solution. They were sectioned at 6 μ and stained by the periodic acid Schiff technique (PAS).

Observations

Vagina grown in natural and defined medium. Before explantation the vaginal epithelium of young, sexually immature mice consists of 2 to 3 rows of small undifferentiated cells and a superficial layer of flat or cuboidal elements filled with a strongly PAS-positive secretory material (FIGURE 1).

In culture, this original epithelium was rapidly shed and replaced by new squamous keratinizing cells. A more detailed examination of the changes showed that shortly after explantation the reserve cells near the basal membrane began to proliferate and push the original epithelium outward; they became stratified into basal, transitional, and precornified cells and formed keratin on the second or third day of growth (FIGURE 2).

These changes were identical in both the natural and the defined medium,

* Supplied by Menformon, Organon Laboratories, London, England.

an indication that under the experimental conditions used the squamous development was independent of the nutritional environment of the tissue.

The effect of radiation on explants in natural medium. In explants grown in natural medium-radiation profoundly modified the spontaneous squamous

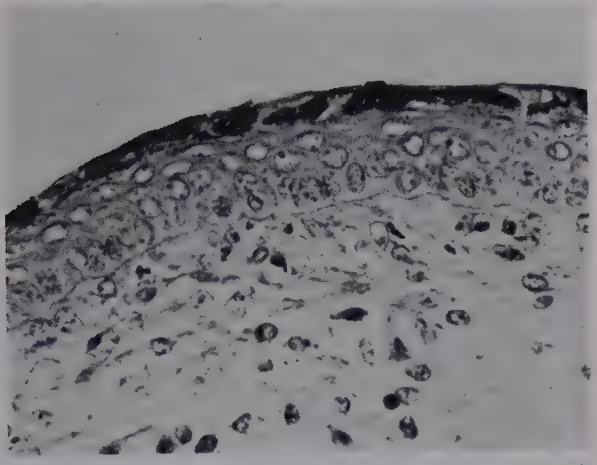


FIGURE 1. Section through a vagina of a two-week-old mouse before explantation, showing two to three rows of immature cells and a superficial layer of flat secretory elements. Periodic acid Schiff after diastase digestion. PAS. ×500.

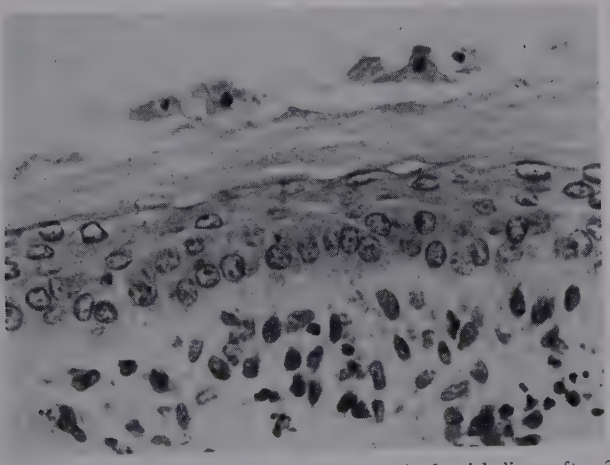


FIGURE 2. Section through an explant of mouse vaginal epithelium after 3-days growth in normal medium. Note squamous transformation with formation of keratin. PAS. ×500.

development seen in the unirradiated controls and inhibited keratinization. Moreover the original epithelium in irradiated explants remained viable and merged with the newly formed cells underneath.

The radiation effect resulted in 2 slightly different versions of the epithelial structure of the vagina. Thus in some explants the epithelium consisted of

2 to 3 rows of small crowded cells of the basal or reserve-cell type, followed by several rows of cells of an indeterminate character and, finally, the original secretory epithelium (FIGURE 3); in other explants the middle layer of cells was replaced by polyhedral elements (FIGURE 4). In both cases the formation of keratin was completely suppressed.

The influence of the defined medium on the effect of radiation. In cultures kept in the defined medium, radiation failed to influence differentiation and,

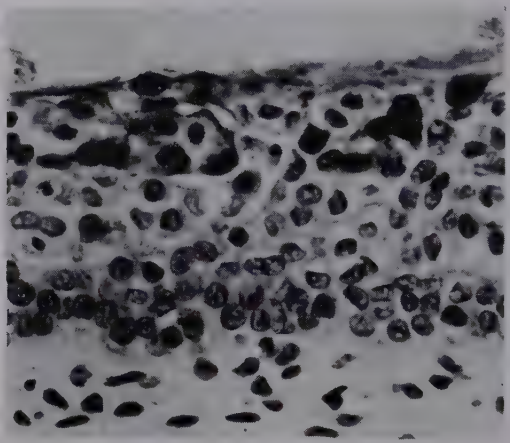


FIGURE 3. Section through a similar explant grown in natural medium for 3 days and exposed to 300-r U. of X rays 1 day after explantation. The cells are of an indeterminate character and keratin is absent. PAS. ×500.

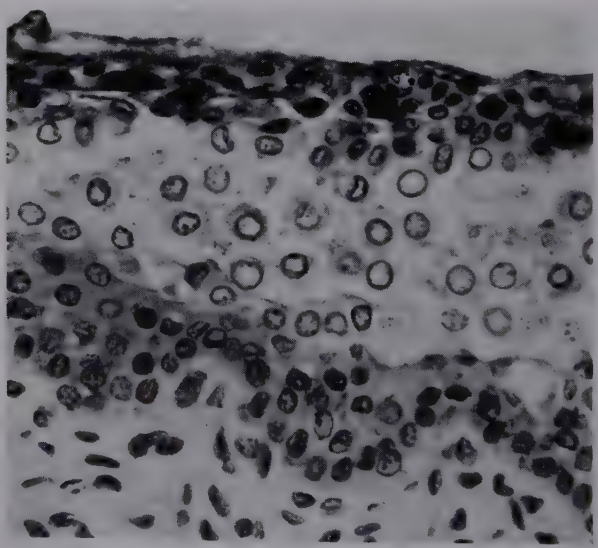


FIGURE 4. Section through a similar explant grown in natural medium for 3 days and exposed to 300-r U. of X rays 1 day after explantation. Note polyhedral cells above the basal layer and at the surface the preservation of the original secretory epithelium. PAS. ×500.

in both control and irradiated explants, the degree and extent of the squamous development and keratinization were identical.

The influence of estrone. In unirradiated explants grown in the presence of the hormone, a squamous development took place similar to that described for cultures without estrone. In both natural and defined medium the reserve cells proliferated near the basal membrane and were then stratified into basal, transitional, and precornified cells and formed keratin. Owing to the stimulation by the hormone, however, both the number of cells and the amount of keratin usually increased so that the epithelium became thicker (FIGURE 5).

Irradiation of estrone-treated explants in natural medium also inhibited keratinization and, as in explants grown without the hormone radiation, produced two different histological patterns. In one, the epithelium consisted

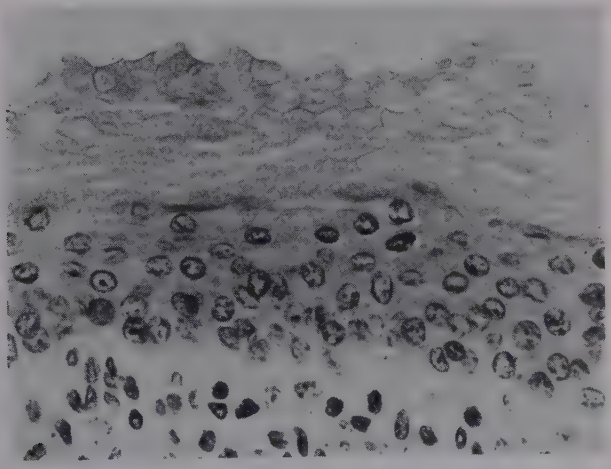


FIGURE 5. Section through a vaginal explant grown for 3 days in medium containing 2 μg./ml. of estrone. Note the greater number of cells and increased amount of keratin as compared with explants grown without the hormone (FIGURE 2). PAS. ×500.

of a layer of basal cells surmounted by many rows of transitional cells covered by a superficial stratum of flat secretory elements (FIGURE 6). The second pattern again showed the appearance of many polyhedral cells above the transitional elements; in contrast to those in explants kept without estrone they lost contact with the cells underneath, the staining reactions of the nuclei varied widely and, in a few cells, shrunken nuclei in a vacuolated cytoplasm could be recognized (FIGURE 7). In such explants the original epithelium usually broke away and formed a separate layer above the polyhedral elements.

Discussion

The results show that, in explants grown in natural medium, irradiation without the intervention of the endocrines changes the direction of differentiation from a squamous toward a mucoid type of structure. Under both conditions there are differences in the extent to which this shift in differentiation is accomplished. Thus in cultures kept without the hormone the original epi-

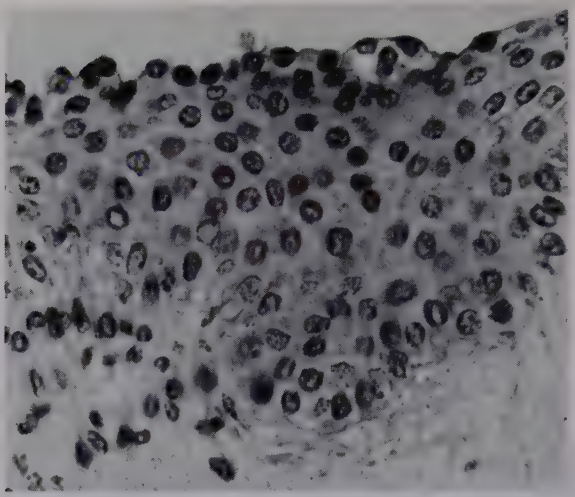


FIGURE 6. Section through a similar explant grown for 3 days in natural medium containing estrone and exposed to 300 r U. of X rays 1 day after explantation. Note the high epithelium consisting of basal transitional and flat secretory cells and the absence of keratin. PAS. ×500.

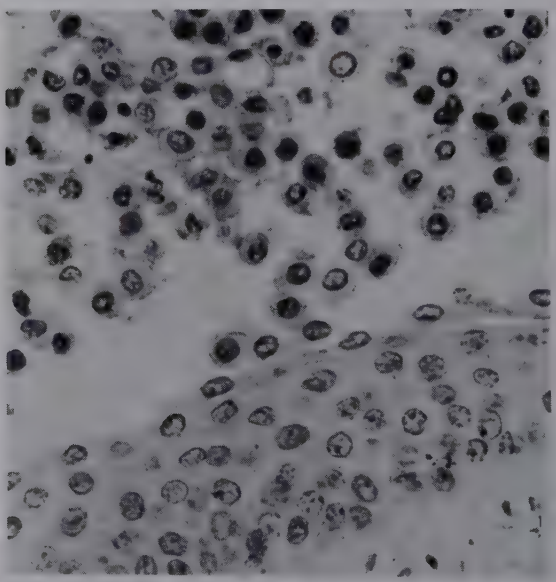


FIGURE 7. Section through a similar explant grown for 3 days in natural medium containing estrone and exposed to 300 r U. of X rays 1 day after explantation. Note polyhedral elements above the transitional cells and absence of keratin. PAS. ×500.

thelium is always preserved, and the stratum between basal cells and original epithelium is composed either of undifferentiated or polyhedral elements. In estrone-treated explants the cells above the basal layer are of the transitional type, that is, they have taken the first step toward a squamous development; the polyhedral elements if present constitute a third layer above them, and their appearance is always associated with a breakdown of the original epithelium. These polyhedral elements occur in a region that in a squamous epithelium is normally occupied by the precornified layer and may be interpreted as variants of precornified cells rendered incapable of forming keratin. Keratin synthesis is usually associated with a shrinkage of nuclei,[22] and the occurrence of such shrunken nuclei in some polyhedral cells, together with the breakdown of the original epithelium and the development of the transitional cells in estrone-treated cultures, suggests an unsuccessful attempt at keratinization under the influence of the hormone.

In this connection it is interesting that the changes produced by radiation in vagina grown in natural medium closely resemble those caused by excess vitamin A. The vitamin, however, acts equally well on explants kept in natural and in defined medium.[20]

The failure of radiation to affect differentiation in explants kept in the defined medium indicates that the nutritional environment of the cultured tissue is an important factor in the realization of the radiation effect and also suggests several possibilities that may shed light on the mechanism whereby radiation acts in the natural medium.

In contrast to the quantitative changes in differentiation in irradiated tumors and intestine, the inhibition of keratinization is unlikely to be linked to an arrest of mitosis. This is supported by Cherry's results,[14] which showed that the mucification in the normal and estrone-treated rat vagina depended partly on the time-relationship between administration of the hormone and exposure to X rays and partly on the concentration of the hormone, but not on the radiation effect on mitosis.

A mutational effect on the nucleus, which in turn might change cytoplasmic synthesis, is also improbable, as such an effect should have been independent of the type of medium used.

It is likely therefore that radiation affects the cytoplasm directly, and the following considerations may offer a possible explanation of how this effect is brought about. There is evidence that disulphide and sulfhydril groups are particularly vulnerable to the effects of ionizing radiation.[21] Cystine and cysteine play a vital role in keratin synthesis, cysteine being an integral part of the "cement" substance built into the fibrous component of keratin.[22] Hence the inhibition of keratinization in the natural medium may be caused by inactivation of the sulfhydril groups by radiation; chemically defined medium on the other hand contains free cysteine in a high concentration, and it is possible that after radiation sufficiently high amounts are left to replace the inactivated cysteine molecules and to allow keratin synthesis to proceed normally.

Cysteine, however, has been found to afford partial protection against radiation,[23,24] and it cannot be entirely ruled out that its presence in the defined medium may prevent the radiation from becoming fully effective.

Another important difference between natural and defined medium that may have a bearing on the response to radiation is that vitamin A is present in natural but absent in chemically defined media. This vitamin plays an essential role in the differentiation of many epithelial tissues, that is, its excess induces mucous metaplasia of squamous epithelium,[25] while A deficiency is followed by squamous transformation of secretory epithelia;[26-29] thus the presence of vitamin A may be a necessary factor for the radiation to become fully effective.

Conclusions

Radiation by a direct effect on the tissue alters the development of the vaginal epithelium from a squamous toward a mucoid type. This qualitative change in the pattern of differentiation does not seem to be either secondary to mitotic inhibition or due to a mutational effect on the nucleus, but is probably caused by an effect on the cytoplasm. This effect may be brought about via an inactivation of substances essential for keratin synthesis and facilitated by the presence of vitamin A in the natural medium.

Summary

The effect of X rays on the differentiation of normal and estrone-treated mouse vaginal epithelium grown as organ culture has been studied.

After explantation into either natural or defined medium, new squamous keratinizing cells displace the secretory epithelium present in the organ *in vivo*.

Exposure to X rays preserves the original secretory epithelium and inhibits the squamous development in explants kept in natural medium, but fails to do so in those kept in defined medium.

Addition of estrone to either natural or defined medium increases the cell number and the amount of keratin in the new squamous epithelium.

Irradiation of estrone-treated cultures inhibits the squamous differentiation of tissue grown in natural medium, but in some explants there is an unsuccessful attempt at keratinization under the influence of the hormone.

In defined medium containing estrone, irradiation also fails to influence the squamous development.

The mechanism of radiation action is discussed in the light of the difference of effect seen in the two media.

Acknowledgments

I thank Honor B. Fell and Alfred Glücksmann for advice and constructive criticism in the preparation of this manuscript; Gladys Gittins for skilled technical assistance; Harold Hignell for help with the irradiation of the cultures; and George Lenney who made the microphotographs.

References

1. Price-Jones, C. & T. C. Mottram. 1914. A contribution to the study of *in vitro* cultures of mouse carcinoma and rat sarcoma. Arch. Middlesex Hospital, 13th Cancer Report. : 21–33.
2. Strangeways, T. S. P. & H. E. H. Oakley. 1923. The immediate changes observed after exposure to soft X rays while growing *in vitro*. Proc. Roy. Soc. B. **95:** 373–381.

3. Spear, F. G. 1931. Immediate and delayed effects of radium on tissue cultures *in vitro*. Brit. J. Radiol. **4:** 147–164. The effect of spaced radiation on tissue cultures *in vitro*. Proc. Roy. Soc. B. **110:** 224–234.
4. Paterson, E. 1944. The time and intensity factor in X-ray irradiation. Brit. J. Radiol. **17:** 25–30.
5. Lasnitzki, I. 1940. The effect of X rays on cells cultivated *in vitro*. Brit. J. Radiol. **13:** 279–284.
6. Lasnitzki, I. 1943*a*. The effect of X rays on cells cultivated *in vitro*. Recovery Factor. Brit. J. Radiol. **16:** 61–67.
7. Lasnitzki, I. 1943*b*. The response of cells *in vitro* to variations in X ray dosage. Brit. J. Radiol. **16:** 137–141.
8. Lasnitzki, I. 1947. A quantitative analysis of the direct and indirect action of X radiation on malignant cells. Brit. J. Radiol. **20:** 240–247.
9. Glücksmann, A. 1941. Preliminary observations on the quantitative examination of human biopsy material taken from irradiated carcinomata. Brit. J. Radiol. **14:** 187–197.
10. Friedman, N. B., J. A. Sargent & E. Drutz. 1955. Certain effects of irradiation and chemotherapy on cellular division and differentiation. Cancer Research. **15:** 479–84.
11. McGrath, R. A. & C. C. Congdon. 1959. X-ray induced abnormal differentiation of epithelium of the small intestine in the mouse. Internat. J. Radiation Biol. **1:** 80–85.
12. Friedman, M. & J. W. Hall. 1950. Radiation-induced squamous cell metaplasia and hyperplasia of the normal mucus glands of the oral cavity. Radiology. **55:** 848–51.
13. Glücksmann, A. 1952. The response of human tissue to radiation with special reference to differentiation. Brit. J. Radiol. **25:** 38–43.
14. Cherry, C. B. 1956. The modification by irradiation of the estrogen effect on the cervicovaginal epithelium of castrated rats. Brit. J. Radiol. **30:** 239–46.
15. Weiss, P. & R. James. 1955. Skin metaplasia *in vitro* induced by brief exposure to vitamin A. Exptl. Cell Research Suppl. **3:** 381–94.
16. Fell, H. B. & R. Robison. 1929. The growth, development and phosphatase activity of embryonic avian femora and limb buds cultivated *in vitro*. Biochem. J. **23:** 767–84.
17. Shaffer, B. M. 1956. The culture of organs from the embryonic chick on cellulose acetate fabric. Expth. Cell Research. **11:** 244–48.
18. Chen, J. M. 1954. The cultivation in fluid medium of organised liver, pancreas and other tissues of foetal rats. Exptl. Cell Research. **7:** 518–529.
19. Healy, G. M., D. C. Fischer & R. C. Parker. 1955. Nutrition of animal cells in tissue culture. X. Synthetic medium No. 858. Proc. Soc. Exptl. Biol. **89:** 71–77.
20. Lasnitzki, I. Effect of excess vitamin A on the normal and estrone-treated mouse vagina grown in chemically defined medium. E. C. R. In press.
21. Pihl, A. & L. Eldjarn. 1958. Pharmacological aspects of ionising radiation and of chemical protection in mammals. Pharmacol. Rev. **10:** 437–74.
22. Bern, H. A., M. Alfert & S. M. Blair. 1957. Cytochemical studies of keratin formation and of epithelial metaplasia in the rodent vagina and prostate. J. Histochem. & Cytochem. **5:** 105–19.
23. Patt, H. B., E. B. Tyree, H. L. Straube & D. E. Smith. 1949. Cysteine protection against X irradiation. Science. **110:** 213–14.
24. Langendorff, H., R. Koch & U. Hagen. 1954. Untersuchungen über einen biologischen Strahelenschutz. VIII. Zur Spezifität des Cystein und verwandter Sulfhydrilkörper beim Straheenschutz. Straheentherapie. **95:** 238–50.
25. Fell, H. B. & E. Mellanby. 1953. Metaplasia produced in cultures of chick ectoderm by high vitamin A. J. Physiol. **119:** 470–88.
26. Wolbach, S. B. & P. R. Howe. 1925. Tissue changes following deprivation of fat soluble A vitamin. J. Exptl. Med. **42:** 753–78.
27. Wolbach, S. B. & P. R. Howe. 1928. Vitamin A deficiency in the guinea pig. Arch. Pathol. Lab. Med. **5:** 239–53.
28. Bern, H. A. 1952. Alkaline phosphatase activity in epithelial metaplasia. Cancer Research. **12:** 85–91.
29. Bern, H. A. 1954. Histology and chemistry of keratin formation. Nature. **174:** 509–11.

CHROMOSOMAL STUDIES ON IRRADIATED LEUKOCYTES *IN VITRO**

Y. Ohnuki,† A. Awa,† C. M. Pomerat
Pasadena Foundation for Medical Research, Pasadena, Calif.

Introduction

In 1956 Tjio and Levan[1] employing the tissue culture method reported the human chromosome number to be 46. Since then many workers have confirmed this finding.[2-6] A standard nomenclature of chromosomal morphology was agreed upon in 1960.[7] Recently culture methods for observing human chromosomes in leukocytes have been greatly improved by Hungerford, and Nowell and his co-workers.[8-10] It is now relatively simple to make reliable preparations that allow detailed analyses necessitating only small amounts of peripheral blood. This technique should facilitate research in several fields, including (1) human cytogenetics, (2) the assessment of clinical response to therapeutic agents, (3) toxicology, (4) radiobiological mechanisms, and (5) the phenomenon of cell differentiation. It is urgent therefore to establish a base line regarding the chromosome morphology of human leukocytes in primary culture treated by this method.

Among the many papers written regarding the effects of irradiation on living animal cells,[11,12] several workers reported on the radiosensitivity of mammalian somatic cells *in vitro* in relation to morphologic studies of chromosomes or to giant cell formation.[13-18]

In the present study, chromosomal analyses of blood cells after short-term culture have been compared with the aberrations induced by irradiation from a cobalt-60 source, in the hope of contributing to the studies that have been cited.

Materials and Methods

Two samples of from 20 to 40 ml. of peripheral blood were obtained from each of 3 individuals in good health, 2 male and 1 female. Leukocytes were separated from heparinized peripheral blood with phytohemagglutinin (PHA),‡ employing slight modifications of the methods introduced by Nowell and Hungerford.[8-10]

For the cultivation of the 4 specimens from the male donors, Bacto-Phytohemagglutinin M (mitosis or muco)-Code 0528, Control No. 442691, was employed. For the later experiments on 2 specimens from the female donor, PHA M-Code 0528, Rx 11734, was used. Both were rehydrated with Gey's balanced salt solution. Each milliliter of the rehydrated solution contained 10 mg. of PHA. Further purification to eliminate the polysaccharide fraction

* The work described in this paper was supported in part by funds provided under Contract AF 41(657)-357 with the School of Aviation Medicine, United States Air Force, Randolph Air Force Base, Tex.

† Fellows of the Tobacco Industry Research Committee, New York, N.Y. Permanent address: Zoological Institute, Hokkaido University, Sapporo, Japan.

‡ Supplied by Difco Laboratories, Detroit, Mich.

has resulted in a product known as Bacto-Phytohemagglutinin P (purified or protein)-Code 3110, Control No. 120184, which has 25 to 50 times greater capacity to agglutinate erythrocytes but, in our hands, it has proved less useful for the cultivation of human leukocytes than the Bacto-Phytohemagglutinin M-Code 0528, No. 442691.

To each 10 ml. of blood, 0.05 ml. of heparin (heparin sodium, 1000 U.S.P. U./ml.*) and 0.15 to 0.2 ml. of PHA were added. After mixing the PHA and blood thoroughly in 15 ml. conical centrifuge tubes the contents were allowed to stand for about 60 min. at approximately 4° C. in ice water, then were centrifuged gently (250 to 300 rpm for 10 min.) to avoid the formation of a buffy coat, and to collect the maximal amount of supernate containing the greatest number of leukocytes. Usually about 2 ml. of plasma was obtained from 10 ml. of blood, with a variation of from 1 to 5 ml. By careful aspiration, 1.5 to 2.0 ml. of supernate was transferred into each T-30 flask. Eagle's synthetic medium with 10 per cent inactivated horse serum, 100 U. of penicillin/ml., and 100 gamma of neomycin/ml. was added so that the seeding had a concentration of approximately 1.0×10^5 cells/ml. in a final volume of 8 to 10 ml. The ratio of supernate to medium was approximately 1:3 or 1:4. This gave a culture depth of approximately 3.0 mm. The cultures were incubated without agitation at 37° C. The cells settled and grew on the bottom of the flasks forming typical colonies (FIGURE 1*a*).

On the fourth day 1 flask of sister cultures was exposed to 400 r of gamma irradiation from a cobalt-60 source with a dosage rate of 47.4 r/min. (or 45.9 r/min.) at the focal distance of 50 cm. for 8 min. 25 sec. (or 8 min. 42 sec.). The flask containing the original medium was placed cell-side down at the center of the 100 $cm.^2$ radiation field with a distance of 50 cm. from the source to the bottom surface. After irradiation the flask was reincubated without change of medium until fixation.

On the sixth day, approximately 45 hours after irradiation, sister cultures were pretreated by adding 0.1 ml. of a 1:10,000 dilution colchicine per 10 ml. of medium for a 3-hour period, and then the cells were harvested from the bottom of the flasks by scraping with a rubber policeman. After centrifugation at 1200 rpm for 5 min. the supernate was decanted and the cells were resuspended in a hypotonic solution consisting of sodium citrate, 1.12 per cent in distilled water, at room temperature for 10 min. The cells were then fixed with freshly prepared 1:3 acetic alcohol (Carnoy's fluid) for about 20 min. Staining was achieved by replacing the fixative with 50 per cent acetic acid for a few minutes, removing all but 3 times the cell volume, and adding 1 or 2 drops of acetic orcein (2 mg. orcein in 100 ml. of 50 per cent acetic acid) per 0.1 ml. of cell suspension. After mixing gently, a small drop of the cell suspension was placed on a clean slide, mounted with a cover slip (7/8 inches square, No. 1 thinness), and squashed with very firm thumb pressure between pieces of blotting paper. The edges of the cover slip were sealed with a mixture of equal parts paraffin and Canada balsam.

For observation, phase-contrast microscopy was used with a Zeiss 100×

* From Abbott Laboratories, North Chicago, Ill., or from The Upjohn Co., Kalamazoo, Mich.

dark contrast objective in combination with a Zeiss 8× eyepiece. Photomicrographs were taken by means of the phase-contrast microscope with a 97× (AO) dark-contrast objective and a Leitz 10× Periplan eyepiece. A camera lucida was used to make exact drawings of the chromosomes. For idiogram analyses, individual chromosomes were cut from photographs and drawings and aligned according to size and the position of the centromeres.

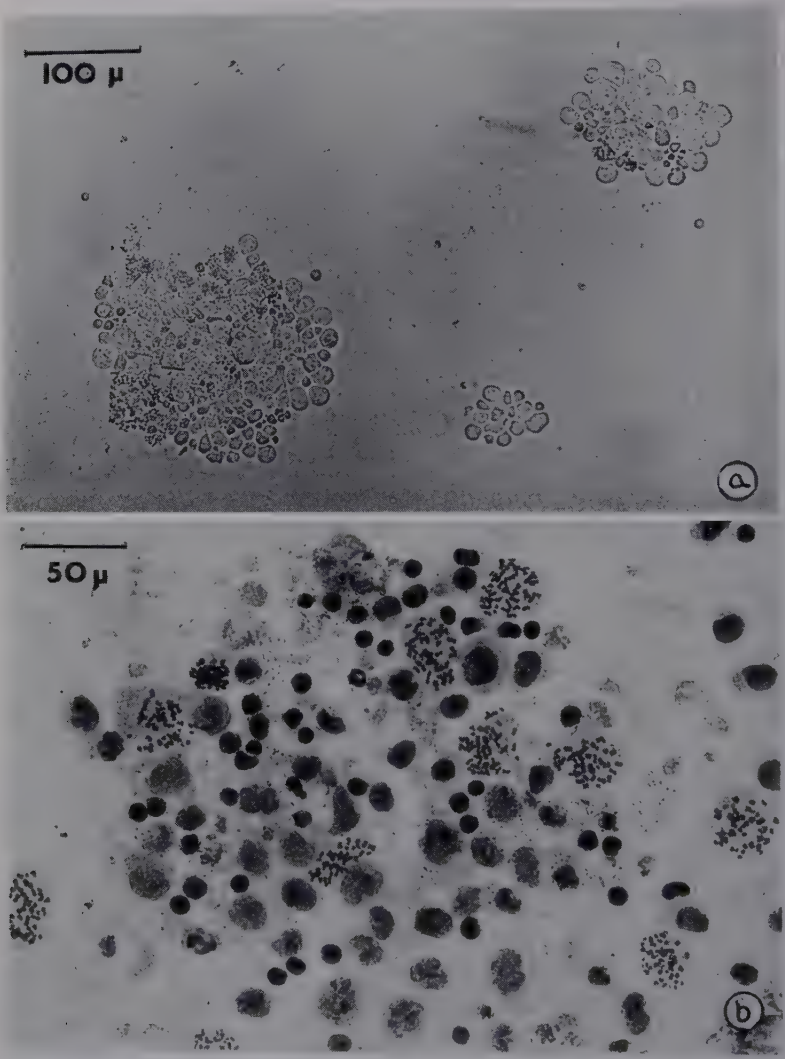


FIGURE 1. (*a*) Five-day culture showing typical colonies (sample P2); and (*b*) cultures fixed on the sixth day, treated with colchicine, Carnoy's fixation, acetic orcein staining (P2). The scale of magnification is given in the upper left area of this and of each succeeding figure representing cellular elements.

Results

After preliminary experiments a dosage of 400 r was selected, since it produced about 50 per cent of metaphase plates showing at least 1 chromosome aberration 48 hours after irradiation, a convenient figure for comparative studies. Two samples of peripheral blood were obtained from each of 3 individuals in good health, 2 male and 1 female (R1, R2, P1, P2, D1, and D2).

TABLE 1
SUMMARY OF THE DISTRIBUTION OF PLOIDY IN CONTROL AND IRRADIATED HUMAN LEUKOCYTES

	Assessment of ploidy							
	Control			Forty-eight hours after 400 r cobalt-60				
	2n	4n	Total	2n	3n	4n	4n<	Total
R1 ♂	69 (100.0)	—	69	105 (96.3)	1 (0.9)	2 (1.8)	1 (0.9)	109
R2	3803 (99.9)	2* (0.1)	3805	392 (91.8)	1 (0.2)	33† (7.7)	1 (0.2)	427
P1 ♂	1037 (100.0)	—	1037	23 (92.0)	—	2‡ (8.0)	—	25
P2	2998 (99.9)	2 (0.1)	3000	463 (92.6)	3 (0.6)	33§ (6.6)	1 (0.2)	500
D1 ♀	1524 (99.9)	1 (0.1)	1525	174 (94.1)	2 (1.1)	9¶ (4.9)	—	185
D2	734 (99.4)	1 (0.1)	735	516 (95.8)	4 (0.7)	17‖ (3.2)	2 (0.4)	539
Total	10165 (99.9)	6 (0.1)	10171	1673 (93.7)	11 (0.6)	96 (5.4)	5 (0.3)	1785

The following footnotes include endoreduplication cells:
* 1 cell.
† 26 cells.
‡ 1 cell.
§ 5 cells.
¶ 1 cell.
‖ 9 cells.

In both control and irradiated cases, rough counts for ploidy determination were performed by low-power scanning (FIGURE 1*b*) and, with oil immersion, exact counts for chromosome number were accomplished using simple drawings.

Control series. As shown in TABLE 1, there were 10,171 cells checked of which 10,165 (99.9 per cent) belonged in the diploid range. Only 6 cells (0.1 per cent) were in the tetraploid range. One of these showed endoreduplication of chromosomes. The number of cells examined varied in each case because of differing conditions of culture; however each donor showed exactly the same percentage (0.1 per cent) of tetraploids (TABLE 2).

Exact chromosome counts of each sample were done by phase-contrast microscopy on at least 30 well-spread metaphases with intact cytoplasm. A total of 150, 160, and 170 metaphases were examined from each donor, R, P, and D respectively (FIGURES 2*a* and *b*; FIGURES 4*a* to *d*; and TABLE 3). Only those cells were accepted that showed a chromosome number that could be accurately determined, except in the case of tetraploids. The predominant chromosome number was 46 in all cases. A detailed description of the results in each case follows.

Case 1 (R1, R2): white male, age 34. In the first sample, 30 metaphases containing 46 chromosomes were observed. Three metaphases of the second sample showed 47, 48, and 88 chromosomes (endoreduplication). One endoreduplication having 92 chromosomes was observed but it is not shown in the tables (FIGURE 3*a*).

TABLE 2
SUMMARY OF THE CHROMOSOME PLOIDY DISTRIBUTION IN CONTROL AND IRRADIATED HUMAN LEUKOCYTES EXPRESSED AS PERCENTAGES

	Assessment of ploidy in per cent					
	Control		Forty-eight hours after 400 r cobalt-60			
	2n	4n	2n	3n	4n	4n<
R ♂	99.9	0.1	92.7	0.4	6.5	0.4
P ♂	99.9	0.1	92.5	0.6	6.7	0.2
D ♀	99.9	0.1	95.5	0.6	3.6	0.3
Average	99.9	0.1	93.7	0.6	5.4	0.3

Case 2 (P1, P2): white male, age 55. In the first sample, 60 metaphases with 46 chromosomes were observed. Of 100 metaphases in the second sample, 1 contained 47 chromosomes and 1 contained 50. There were 2 endoreduplications (92, 95±) and 1 tetraploid (92±) that are shown in FIGURE 13.

Case 3 (D1, D2): white female, age 23. Of 50 metaphases in the first sample, the predominant chromosome number was 46. One had 47 chromosomes and 1 showed the hypodiploid chromosome number 33, such as Nowell and Hungerford[10] reported (FIGURE 3*b*). From the second sample, 120 metaphases were observed that all showed 46 chromosomes.

Three idiogram analyses were done from each donor by camera lucida drawings or photomicrographs of well-spread metaphase plates. Of these, 2 are represented in FIGURE 5. Idiograms were made according to the standard system of nomenclature agreed upon by the international Human Chromosome Study Group. All idiograms demonstrating 46 chromosomes belonged to the standard pattern. Detailed morphologic description of individual chromosomes, particularly the sex elements, the satellites on autosomes 13, 14, and 21, and aneuploid cells, will be published in a future report.

Irradiation series. In comparison with the controls, the number of poly-

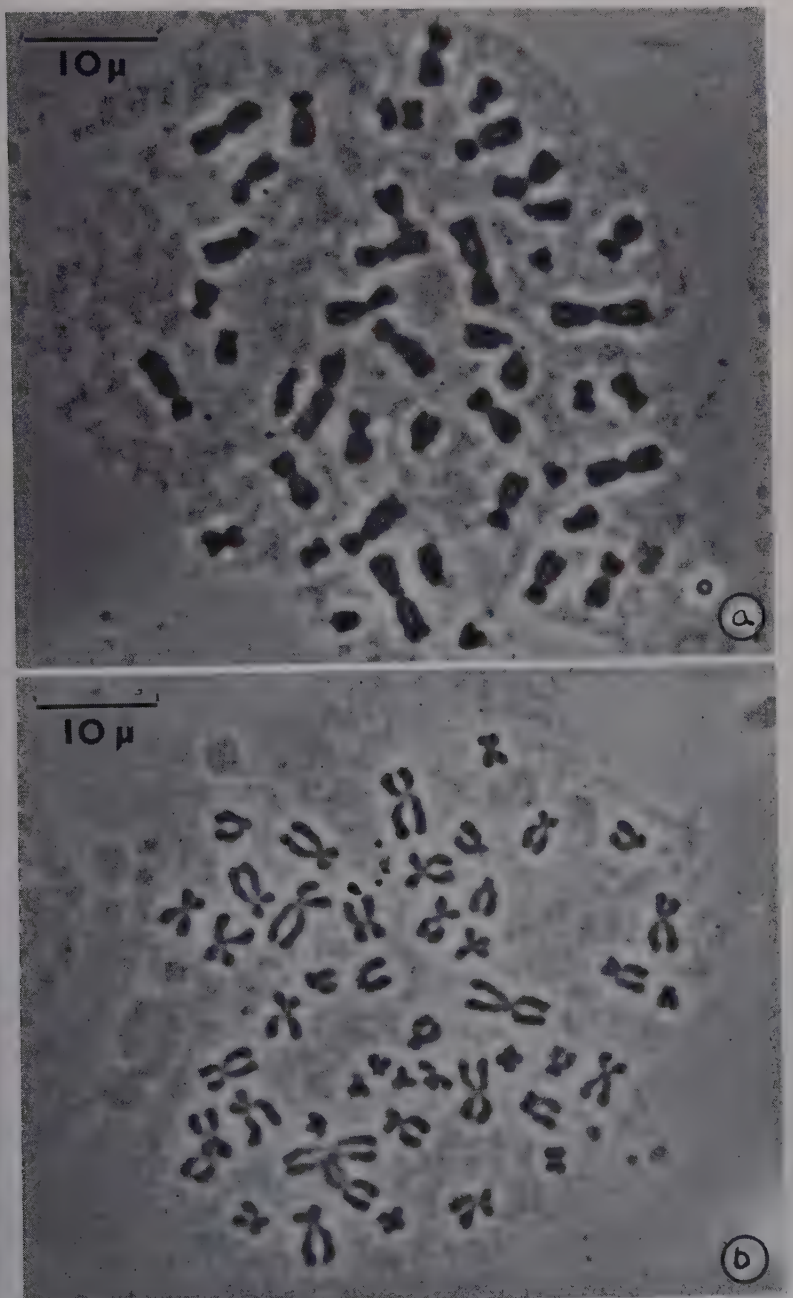


FIGURE 2. (*a*) Metaphase of normal male (P2); and (*b*) metaphase of normal female (D2).

ploid cells increased remarkably after irradiation. There were 96 of 112 (85 per cent) that fell into the range of tetraploidy, or 5.4 per cent of the total irradiated metaphases (TABLE 1). Of these, 42 cells (43.8 per cent) showed endoreduplication. The distribution of tetraploid cells in each sample fluctuated to some extent, ranging from 1.8 to 8.0 per cent; however, each donor showed a similar percentage level (TABLE 2).

Triploid and polyploid cells containing more than 4n chromosomes were also observed. The occurrence of these cells was less than 1 per cent in almost all cases.

Morphologic analyses of chromosome aberrations were performed on irradiated metaphase cells. As shown in TABLE 3, of 226 well-spread metaphases

TABLE 3
MODAL VALUE OF CHROMOSOMES IN CONTROL AND SUMMARY OF THE CHROMOSOMAL ABERRATIONS INDUCED BY IRRADIATION

	Control at 6 days		Forty-eight hours after 400 r cobalt-60						
	N	Mode	N	Chromosome injuries					
				Total	D-a	B and D	E	S	P
R1 ♂	30	46	15	4	3	1	—	—	—
R2	120	46*	50	28	1	21	5	—	1
P1 ♂	60	46	5	4	2	1	1	—	—
P2	100	46†	66	34	14	10	3	1	6
D1 ♀	50	46‡	45	30	4	23	2	1	—
D2	120	46	45	28	2	20	3	2	1
Total	480	—	226	128	26	76	14	4	8

Key: D-a, dicentrics alone; B and D, breakages with or without dicentrics; E, endoreduplication; S, stickiness; and P, polyploidy.
* 46(117), 47(1), 48(1), 88(1) endoreduplication.
† 46(95), 47(1), 50(1), 4n(3) includes 2 endoreduplications.
‡ 46(48), 33(1), 47(1).

observed, 98 appeared unaffected. They were essentially of the same chromosome pattern as the standard idiogram (FIGURES 6*a*, 9*a*, and 11*a*). The average number of unaffected cells was 43.4 per cent, ranging from 35.5 to 50.8 (TABLE 4).

The remainder were affected by irradiation and demonstrated at least 1 chromosomal injury. These abnormalities were tentatively classified into the following 5 categories for the convenience of further analyses: (1) one or more dicentric chromosomes only; (2) chromosome or chromatid breakages, with or without dicentrics; (3) endoreduplication; (4) stickiness; and (5) polyploid cells other than endoreduplication (TABLE 3). Categories 1 and 2 could be grouped together as representing the same type of injury, since both are derived from breakage and reunion. However, a significant number of cells showed only dicentrics so that the 2 categories were separated.

Of these 5 patterns of injuries, chromosomal breakages, combined at times with dicentric chromosomes, were the most frequent, nearly 60 per cent (TABLE

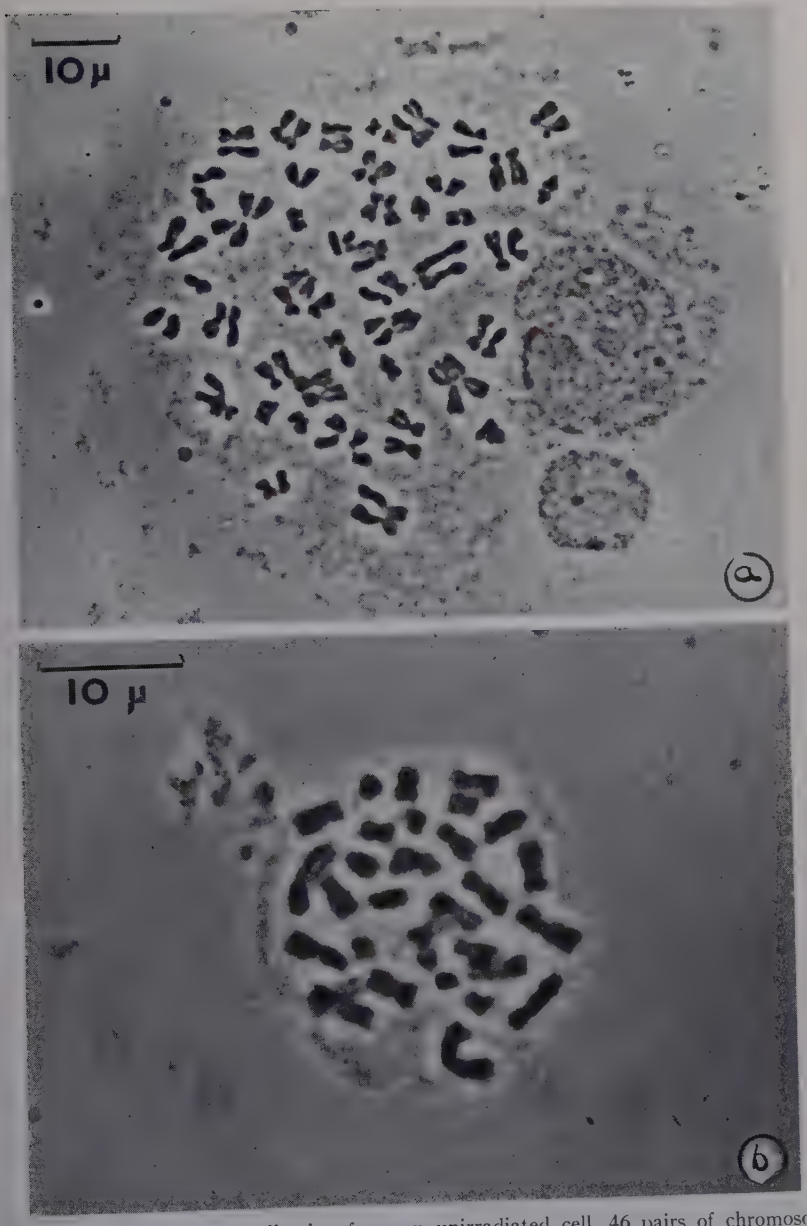


FIGURE 3. (*a*) Endoreduplication from an unirradiated cell, 46 pairs of chromosomes (R2); and (*b*) hypodiploid metaphase with intact cytoplasm from an unirradiated cell, 33 chromosomes (D2).

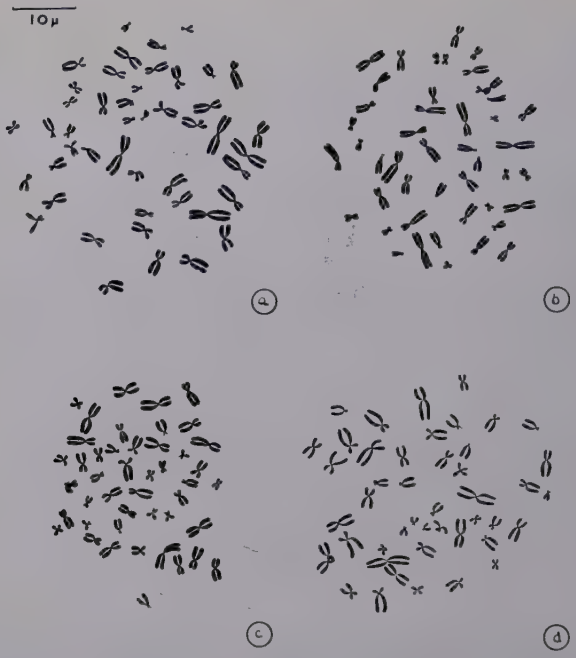


FIGURE 4. (*a*, *b*, *c*, and *d*) Chromosomes of metaphases, camera lucida drawings (R1, P2, D1, and D2).

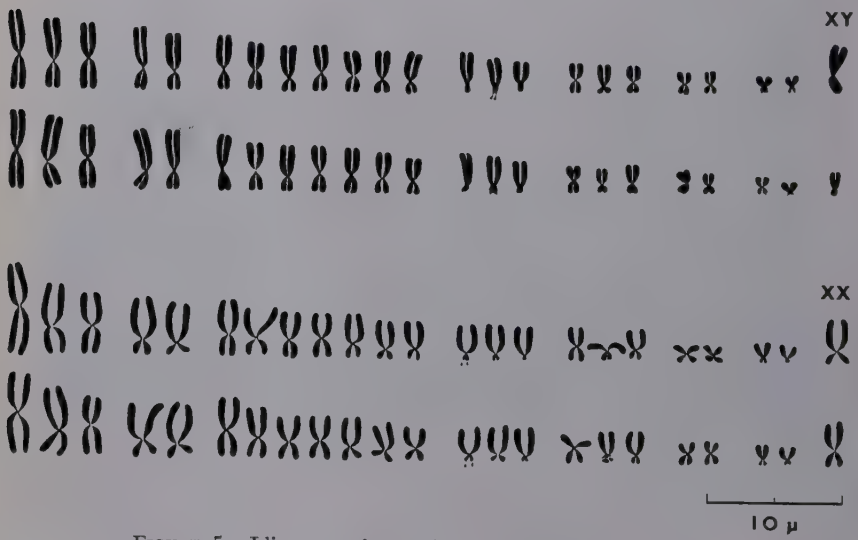


FIGURE 5. Idiograms of metaphases seen in FIGURES 4*b* and *d*.

5). This category was mainly characterized by minute or acentric chromosome fragments (FIGURES 9*b* and 11*p*, *t*, and *v*). Chromosomal translocations and formation of ring elements as well as chromosomes with unusual shapes were rather rare in the present observations (FIGURES 8*b*, 9*b*, 10*a* and *c*, and 11*k* and *u* to *w*).

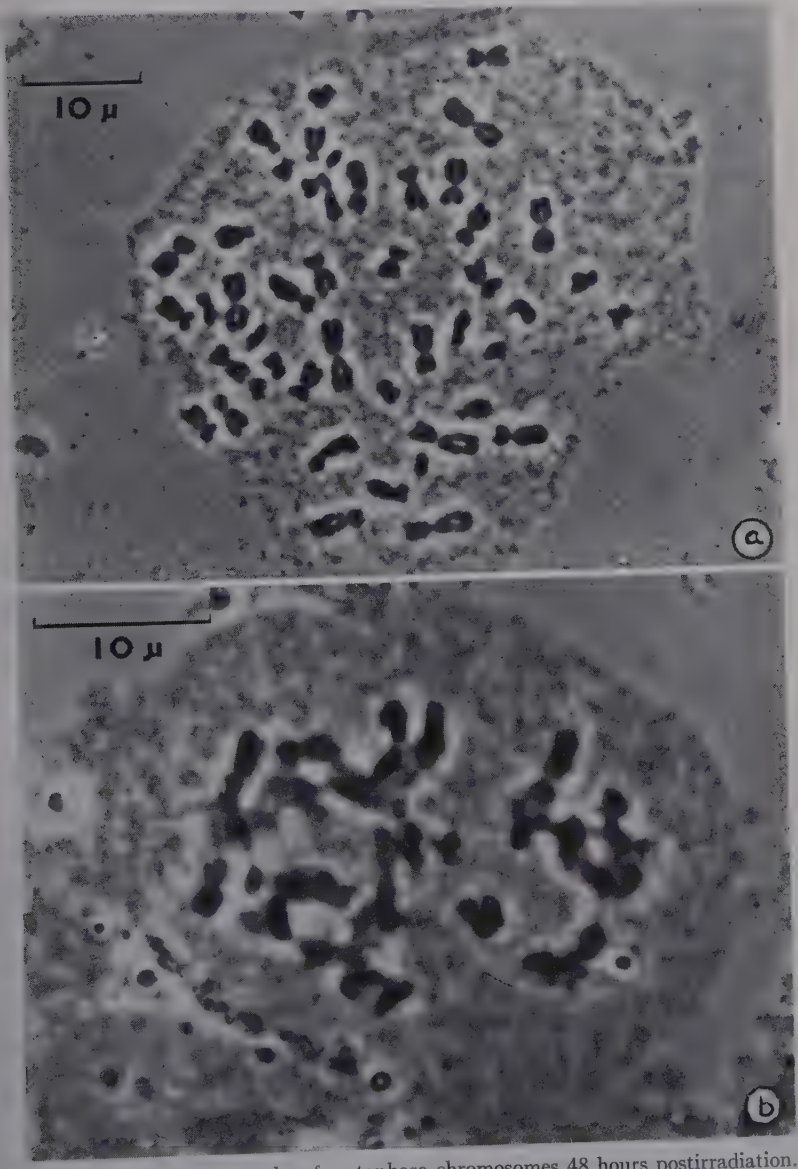


FIGURE 6. Photomicrographs of metaphase chromosomes 48 hours postirradiation. (*a*) Forty-six chromosomes presumably unaffected (R1), see FIGURES 9*a* and 11*a*; and (*b*) metaphase showing marked stickiness (P2).

Category 1, or metaphases with 1 or more dicentric chromosomes only, was seen in over 20 per cent of total injuries (FIGURES 9*c* and *d*, and 10*b*). The number of the dicentric chromosomes per cell in the diploid range varied from 1 to 3. In 1 case (P2), this type of aberration was particularly outstanding. A comparison was made of the longest and shortest chromosomes with the dicentric elements (FIGURE 11*b* to *j* and *l* to *s*). It was noted that there were no specific chromosomes that formed dicentric elements after irradiation.

Endoreduplication was observed in most samples (TABLES 3 and 4; FIGURES 7*b*, 8*a*, and 10*d*). In the majority of these cells, chromatid elements of individ-

TABLE 4
PERCENTAGE OF CELLS WITH CHROMOSOMAL INJURIES

	No. of cells observed	No. of cells unaffected	No. of cells injured
R	65	33 (50.8)*	32 (49.2)
P	71	33 (46.5)	38 (53.5)
D	90	32 (35.5)	58 (64.5)
Total	226	98 (43.4)	128 (56.6)

* Numbers in parentheses represent percentages.

TABLE 5
SUMMARY OF CHROMOSOMAL INJURIES IN PER CENT
HUMAN LEUKOCYTES
Cultivated for 4 days, then treated with 400 r (gamma) fixed 2 days later

	Per cent
Total injuries:	56.6
Dicentrics	20.3
Breakage and dicentrics	59.4
Endoreduplication	10.9
Stickiness	3.1
Polyploidy	6.2

ual chromosomes appeared to be morphologically normal. A few cells with endoreduplications showed various breakage patterns. Stickiness of chromosomes was rare in this experiment (FIGURE 6*b*), being 3.1 per cent of total injuries. No analysis of the time relationship between the mitotic cycle of leukocytes and the time of irradiation and observation is described; however such work is in progress.

Discussion

Since Tjio and Levan[1] reported the human chromosome number to be 46, many workers reexamined their findings in different tissues with improved culture techniques. Chu and Giles,[2] employing surgical biopsies of various tissues, described chromosome numbers and morphology. Makino and Sasaki[5,6] studied the distribution of ploidy and chromosome morphology in

many tissues from 41 human foetuses. Nowell and Hungerford[10] analyzed the chromosomes of normal and leukemic human leukocytes. In the present study human leukocytes were also used to examine ploidy, chromosome number, and morphology.

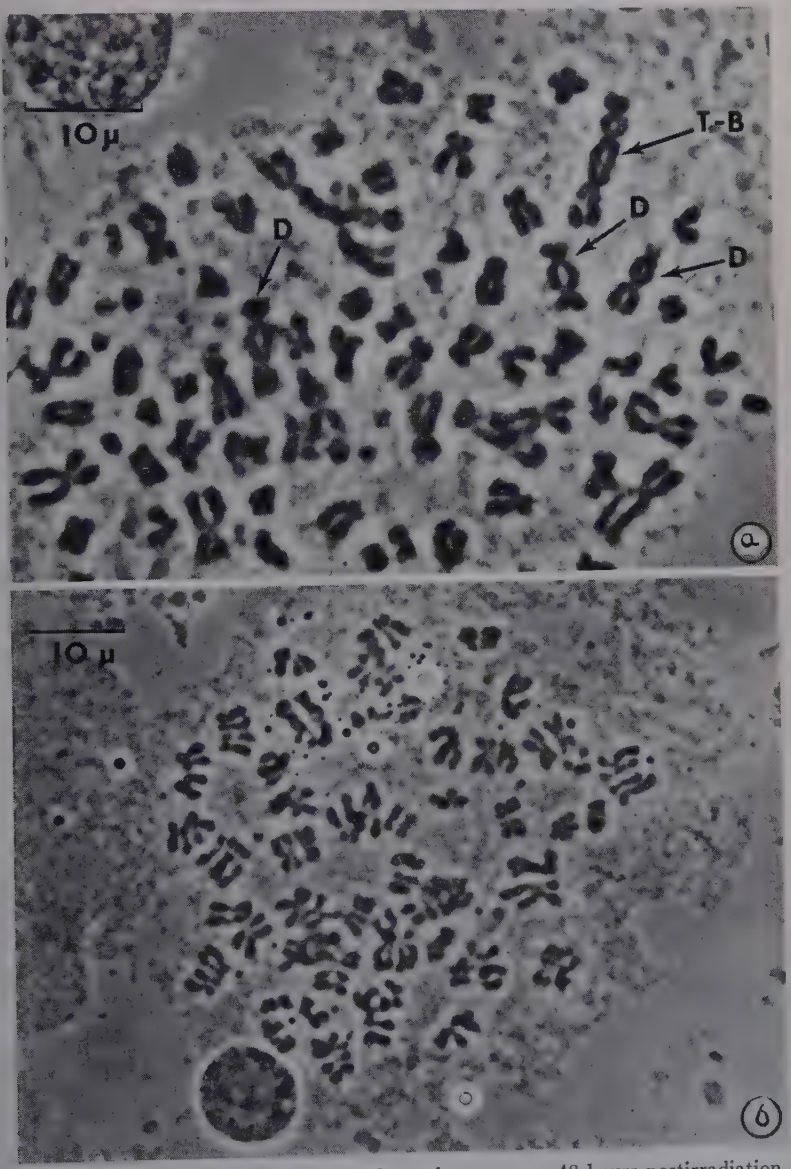


FIGURE 7. Photomicrographs of metaphase chromosomes 48 hours postirradiation (P2). (*a*) Polyploid cell with several dicentrics; *T-B*, tricentric with chromatid breakage; *D*, dicentric; and (*b*) endoreduplication.

In their ploidy determination Tjio and Puck[19] found the tetraploid frequency in 2000 cells to be less than 3 per cent. Nowell and Hungerford[10] reported approximately 2 per cent tetraploids. Makino and Sasaki[5,6] observed 50 tetraploids in 3048 cells examined (1.14 per cent). This differed from our

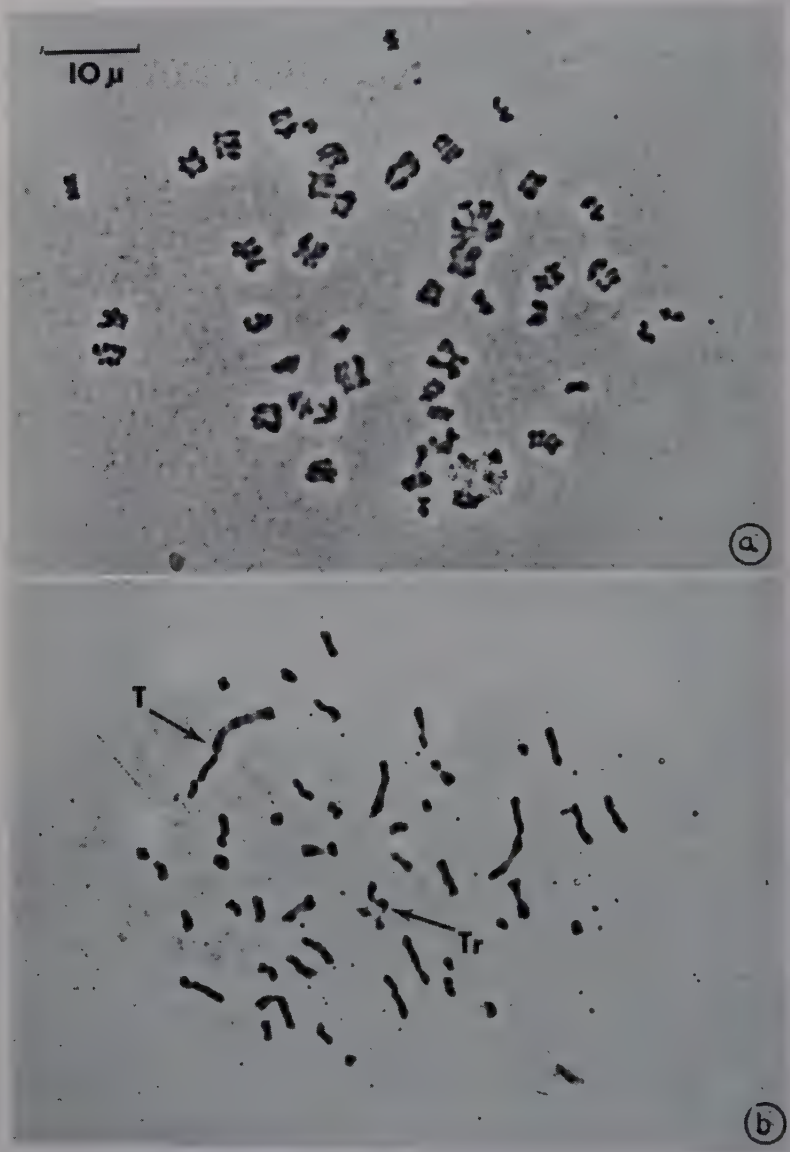


FIGURE 8. Photomicrographs of metaphase chromosomes 48 hours postirradiation (R2). (*a*) Endoreduplication; and (*b*) metaphase showing chromosome breakages; *Tr*, translocation of chromosomes; and *T*, tricentric.

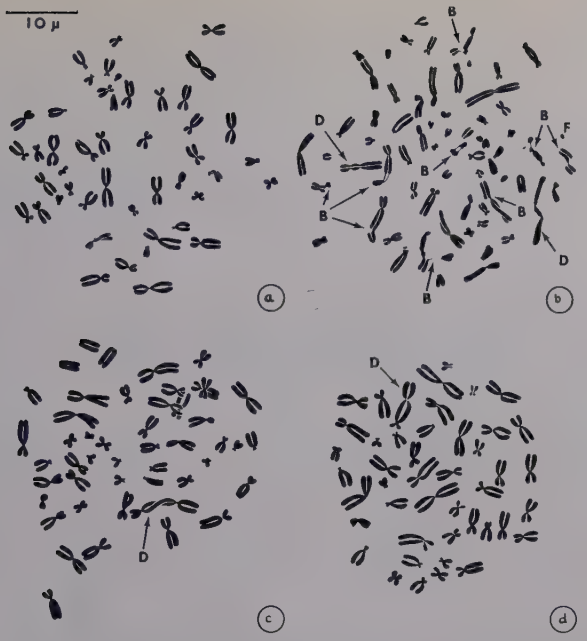


FIGURE 9. Drawings of metaphase chromosomes 48 hours postirradiation. (*a*) Metaphase presumably unaffected (R1) see FIGURE 11*a*; (*b*) metaphase showing breakages (P2); *B*, breakage and translocation; *D*, dicentric; and (*c* and *d*) metaphases each having 1 dicentric (P2).

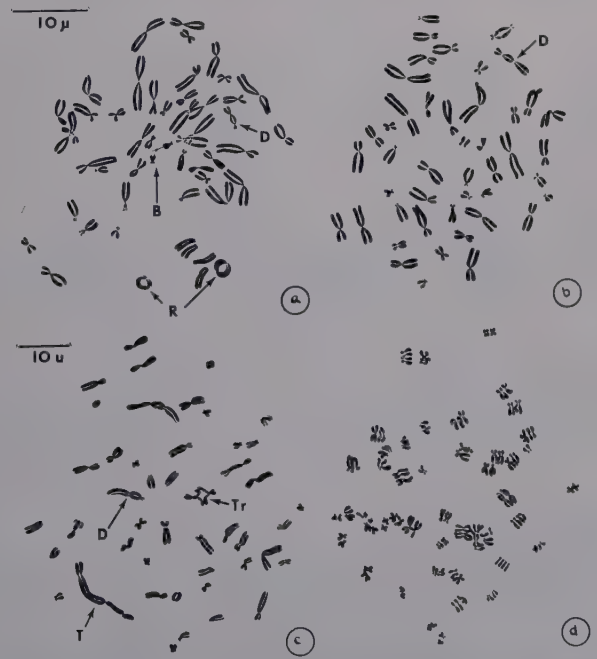


FIGURE 10. Drawings of metaphase chromosomes 48 hours after irradiation (R2). (*a*) Abnormal metaphase; *R*, ring; *D*, dicentric; and *B*, breakage; (*b*) metaphase having 1 dicentric; (*c*) metaphase with various injuries; *Tr*, translocation; *D*, dicentric; and *T*, tricentric; and (*d*) endoreduplication.

results of 0.1 per cent, possibly due to materials, methods, and the number of cells examined (FIGURE 12).

When the chromosome numbers of these 4 studies were compared, all data showed a similar pattern. Nowell and Hungerford[10] reported a higher percentage of indeterminable counts but the variation was so slight (45±, 46±,

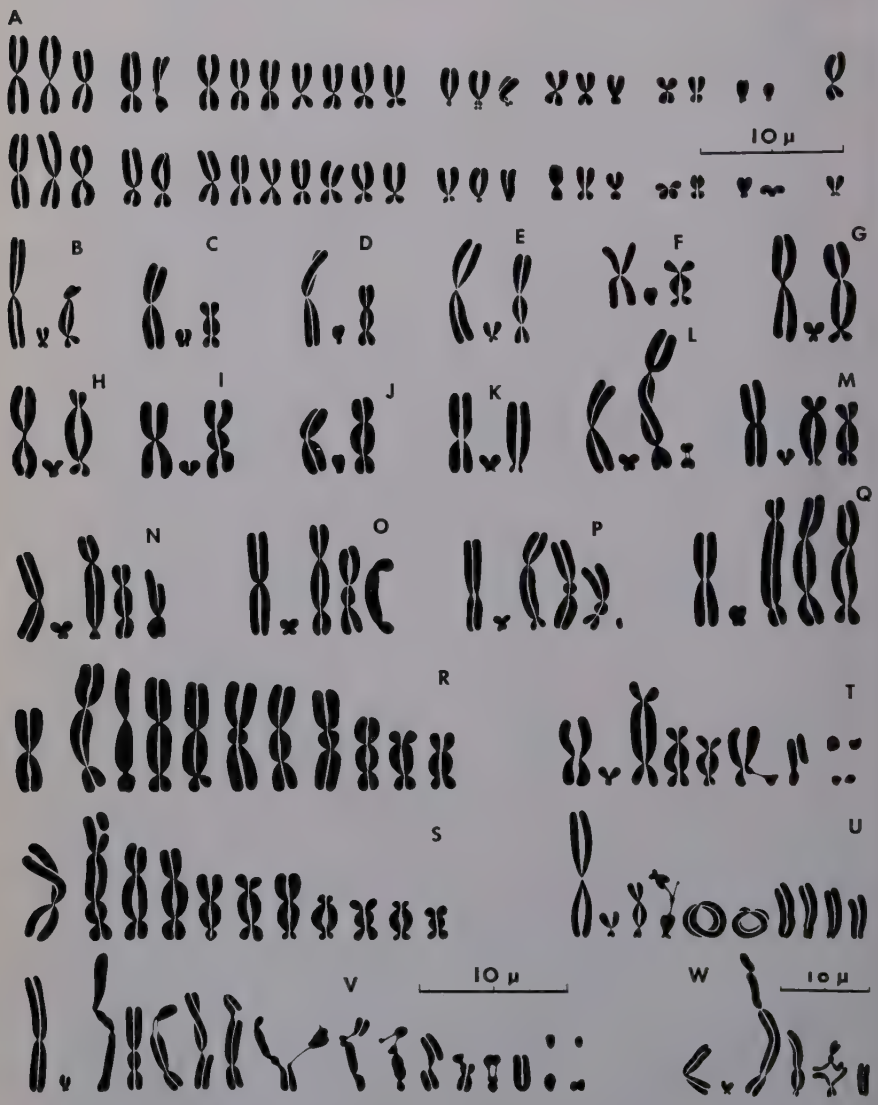


FIGURE 11. Drawings of metaphase chromosomes 48 hours postirradiation (R1, R2, P1, and P2). (*a*) Idiogram from FIGURE 9*a* showing normal pattern; (*b* to *w*) comparison of the longest and shortest chromosomes with abnormal elements: (*b* to *j*) one dicentric; (*k*) unusually long chromosome of rod-shape; (*l* to *s*) 2 to 10 dicentrics; and (*t* to *w*) several patterns of chromosomal aberrations including dicentrics, rings, fragments, and translocations.

47±) that many cells may actually have had 46 chromosomes (FIGURE 13). Slight differences in aneuploidy were observed among these studies, especially in the case of leukocyte cultures, but that could be expected with varying tissue culture conditions.

Both studies of leukocytes showed the very rare occurrence of hypodiploid cells ranging from 10 to 33 chromosomes. Nowell and Hungerford[10] explained the occurrence of at least 4 reported hypodiploid cells as having been caused

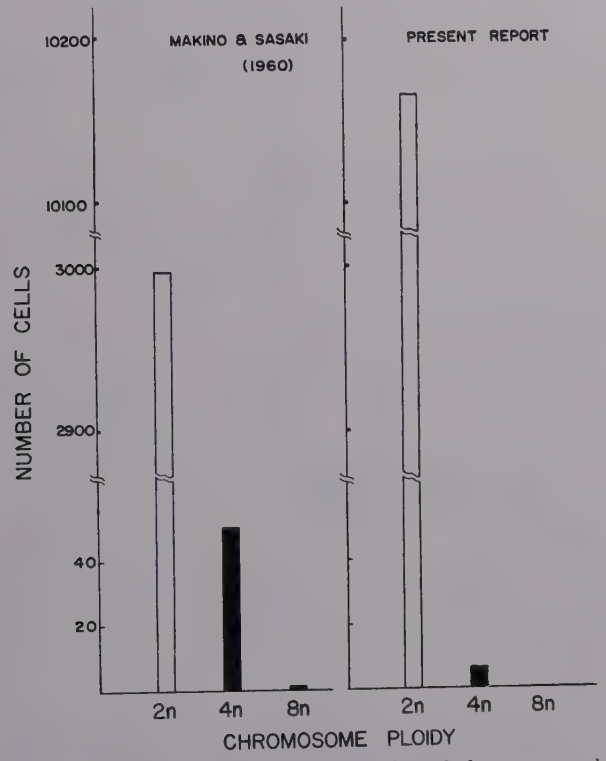


FIGURE 12. Histograms representing the distribution of chromosome ploidy in human somatic cells *in vitro* by Makino and Sasaki[5] and in the present report.

by long-term colchicine (1×10^{-7} *M*) treatment (17 to 19 hours). In our study only 1 hypodiploid cell with 33 chromosomes was observed after treatment with colchicine for 3 hours (FIGURE 3*a*). No somatic pairing in diploids such as Sandberg *et al.*[20] observed in human bone marrow culture was found.

It was noteworthy that in the present experiment there was a significant increase in the percentage of tetraploid cells after irradiation with 400 r. Since the distribution of euploidy was constantly high in unirradiated cells cultivated *in vitro*, the appearance of tetraploid cells could be a valuable criterion for measuring radiation effects.

As a result of detailed morphologic analyses, patterns of chromosome injuries obtained were similar to those found by other workers.[11,12,17,22-24] Of these

injuries chromosomal fragmentations and dicentrics were the most frequent. The former may induce gene mutations or lead to cell death, while the latter occurs not only in irradiated cells[16,18,21] but also in cells treated with some agents[25] or even in unirradiated cell strains *in vitro*, probably due to environmental changes.[26-28] It was of interest that stickiness of chromosomes was less manifest. This abnormality is always found in the early postirradiation

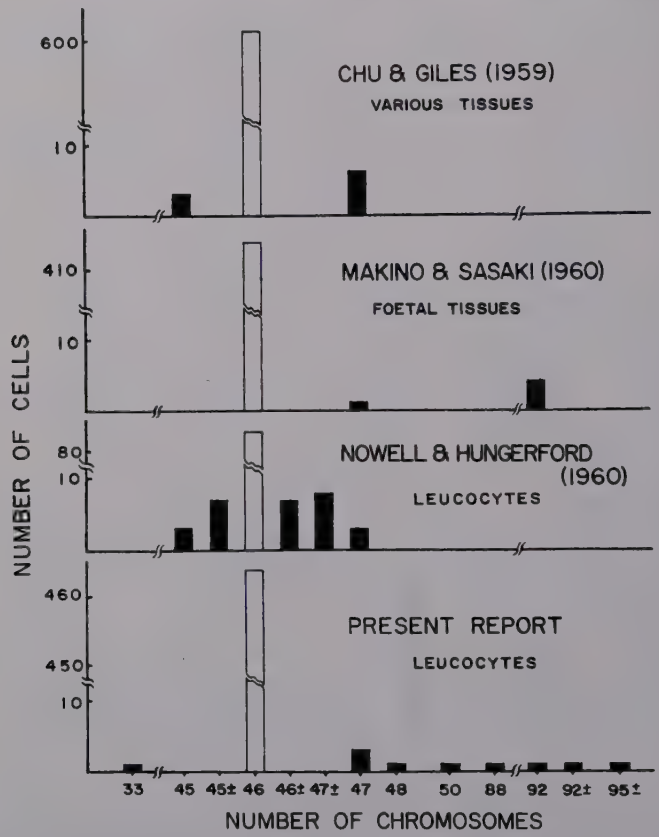


FIGURE 13. Histograms comparing the range of human diploid numbers in recent studies of somatic cells cultivated *in vitro*.

period. Our observations, however, were made of cells 48 hours after irradiation. At that time very few cells exhibited stickiness. In every sample, under the conditions of these experiments, slightly more than one half of the metaphase cells showed at least one chromosomal injury. Although the number and pattern of such irregularities varied from cell to cell, the values obtained were relatively constant.

Using the method of Hungerford *et al.*,[8] Tough and his associates[21] reported chromosomal injuries to human leukocytes from 2 patients with ankylosing spondylitis for several days following X-ray therapy. In the first case chromo-

some fragments were common, and complex abnormalities such as ring elements and dicentrics were seen after treatment with a total skin dose of 1500 rads in 10 equal fractions. The second patient, given a single dose of 250 rads, showed a rise in cells with abnormal chromosomes from 1 per cent preexposure to 22 per cent 1 day postirradiation, reaching a peak of 41 per cent 3 days after treatment.

It is of importance that similar patterns of chromosomal injuries have been demonstrated in our study, although radiation was administered *in vitro*. Comparative studies on damage to leukocytic chromosomes of man and experimental animals irradiated both *in vitro* and *in vivo* are urgently needed. Such quantitative analyses might be of value in the assessment of various therapeutic measures employed in the treatment of malignancies.

Summary

The technique for the cultivation of the human buffy coat with the use of heparinized venous blood treated with phytohemagglutinin has been described. With this method chromosome spreads were made from 6-day cultures using the colchicine-hypotonic sodium citrate-acetic orcein-squash sequence. The range of polyploidy was determined on 2 specimens for each of 3 individuals (2 males and 1 female). Detailed analyses and counts of human chromosomes indicated the predominant distribution to be euploid cells having 46 chromosomes. Idiogram studies showed the same basic pattern demonstrated by previous investigators.

These data served as a basis for comparing results obtained from sister cultures irradiated with 400 r from a cobalt-60 source on the fourth day and fixed on the sixth day. Under these experimental conditions there was a considerable increase, 6.3 per cent, in the number of polyploid cells. Among these, tetraploid cells were the most frequent, being 5.4 per cent. By chromosomal analyses, 56.6 per cent of the cells were found to be damaged. Most injuries were due to chromosomal breakages, especially fragments and dicentrics. Endoreduplication also was noted frequently.

Acknowledgments

Grateful acknowledgment is made to Rosemary Dobson and T. Seto for indispensable aid in the study of the chromosomes and the preparing of the manuscript. C. George Lefeber and William Huff prepared the photographs, and Charles Raiborn, Jr. was responsible for the culture media.

References

1. Tjio, J. H. & A. Levan. 1956. The chromosome number of man. Hereditas. **42:** 1–6.
2. Chu, E. H. Y. & N. H. Giles. 1959. Human chromosome complements in normal somatic cells in culture. Am. J. Human Genet. **11:** 63–79.
3. Ford, C. E., P. A. Jacobs & L. G. Lajtha. 1958. Human somatic chromosomes. Nature. **181:** 1565–1568.
4. Levan, A. & T. C. Hsu. 1959. The human idiogram. Hereditas. **45:** 665–674.
5. Makino, S. & M. Sasaki. 1960. A study of the somatic chromosomes in the Japanese. Jap. J. Genet. **35:** 228–237.
6. Makino, S. & M. Sasaki. 1961. A study of somatic chromosomes in a Japanese population. Am. J. Human Genet. **13:** 47–63.

7. Human Chromosome Study Group. 1960. Proposed chromosome nomenclature. A.I.B.S. Bull. **10**(6): 27–30.
8. Hungerford, D. A., A. J. Donnelly, P. C. Nowell & S. Beck. 1959. The chromosome constitution of a human phenotypic intersex. Am. J. Human Genet. **11:** 215–236.
9. Nowell, P. C. 1960. Phytohemagglutinin: An initiator of mitosis in culture of normal human leucocytes. Cancer Research. **20:** 462–466.
10. Nowell, P. C. & D. A. Hungerford. 1960. Chromosome studies on normal and leukemic human leucocytes. J. Natl. Cancer Inst. **25:** 85–109.
11. Kaufmann, B. P. 1954. Chromosome aberrations induced in animal cells by ionizing radiations. *In* Radiation Biology. **1:** 627–712. A. Hollaender, Ed. McGraw-Hill. New York, N.Y.
12. Lea, D. E. 1955. Actions of Radiations on Living Cells. Cambridge Univ. Press. New York, N.Y.
13. Pomerat, C. M., S. P. Kent & L. C. Logie. 1957. Irradiation of cells in tissue culture. I. Giant cell induction in strain cultures versus elements from primary explants. Ztschr. Zellforsch. **47:** 158–174.
14. Pomerat, C. M., S. P. Kent & L. C. Logie. 1957. Irradiation of cells in tissue culture. II. Cinematographic analyses of cell enlargement and mitotic activity following gamma irradiation at 2000 r and 4000 r. Ztschr. Zellforsch. **47:** 175–197.
15. Puck, T. T. 1958. Action of radiation on mammalian cells. III. Relationship between reproductive death and induction of chromosome anomalies by X irradiation of euploid cells *in vitro*. Proc. Natl. Acad. Sci. **44:** 772–780.
16. Puck, T. T. 1960. *In vitro* studies on the radiation biology of mammalian cells. *In* Progress in Biophysics. **10:** 238–257. Pergamon Press. New York, N.Y.
17. Wakonig, R. & D. K. Ford. 1960. Chromosome aberrations in irradiated cells of Chinese hamster grown in tissue culture. Can J. Zool. **38:** 203–207.
18. Mizutani, M., Y. Ohnuki, Y. H. Nakanishi & C. M. Pomerat. 1961. Changes in modal values of chromosomes after irradiation of human amnion cells. Cancer. Reprinted from Proceedings Conference on Research on the Radiotherapy of Cancer. Am. Cancer Soc., Inc. : 51–69.
19. Tjio, J. H. & T. T. Puck. 1958. The somatic chromosomes of man. Proc. Natl. Acad. Sci. **44:** 1229–1237.
20. Sandberg, A. A., G. F. Koepf, L. H. Crosswhite & T. S. Hauschka. 1960. The chromosome constitution of human marrow in various developmental and blood disorders. Am. J. Human Genet. **12:** 231–249.
21. Tough, I. S., K. E. Buckton, A. G. Baikie & W. M. Court-Brown. 1960. X ray-induced chromosome damage in man. Lancet. **2:** 849–851.
22. Koller, P. C. 1953. The cytological effects of irradiation at low intensities. *In* Symposium on Chromosome Breakage (1952). : 5–22. Thomas. Springfield, Ill.
23. Ohnuki, Y. 1958. Phase cinematography studies on the effects of radiation and chemicals on the cell and the chromosome. I. Types of X-ray induced chromosome abnormalities in grasshopper spermatocytes, with a note on the normal course of the first division as control. J. Fac. Sci. Hokkaido Univ. Ser. VI. **14:** 83–91.
24. Ohnuki, Y. & S. Makino. 1960. Phase cinematography studies on the effects of radiation and chemicals on the cell and the chromosome. II. Formation of anuclear buds, continuation of chromosome stickiness and formation of an accessory nucleus in grasshopper spermatocytes following X irradiation. Texas Repts. Biol. and Med. **18:** 66–74.
25. Nakanishi, Y. H., M. Mizutani & C. M. Pomerat. 1959. Smoke condensates on lung cells in tissue culture with special reference to chromosomal changes. Texas Repts. Biol. and Med. **17:** 542–590.
26. Chu, E. H. Y. & N. H. Giles. 1958. Comparative chromosomal studies on mammalian cells in culture. I. The HeLa strain and its mutant clonal derivatives. J. Natl. Cancer Inst. **20:** 383–401.
27. Hsu, T. C. & O. Klatt. 1958. Mammalian chromosomes *in vitro*. IX. On genetic polymorphism in cell populations. J. Natl. Cancer Inst. **21:** 437–473.
28. Ruddle, F. H., L. Berman & C. S. Stulberg. 1958. Chromosome analysis of five long-term cell culture populations derived from nonleukemic human peripheral blood (Detroit strains). Cancer Research. **18:** 1048–1059.

THE EFFECT OF X IRRADIATION ON THE PROGRESS OF STRAIN U-12 FIBROBLASTS THROUGH THE MITOTIC CYCLE

Helen Harrington

Division of Radiation Biology, Department of Radiology, Western Reserve University, Cleveland, Ohio

The use of cell culture in radiobiological research has facilitated the investigation of the relationship between cytological and biochemical changes which occur following irradiation. Thus cells cultured *in vitro* are able to carry out processes necessary for growth and replication, but after radiation, indirect effects which occur *in vivo* from the liberation of toxic agents or from gross changes in cell populations are eliminated. For the past several years, various manifestations of radiation damage have been studied in strain U-12 fibroblasts in an attempt to elucidate the sequence of events following irradiation. These studies have included the effect on cell replication, on the process of cell division, on the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and on the progress of the cell cycle in general.

Methods

The general procedures that have been used have been described previously.[1] Strain U-12 fibroblasts, isolated by Swim and Parker,[2] have been grown on a glass surface in a chemically defined medium, S-103[3], supplemented with 20 per cent horse serum. Cell multiplication was measured either by counting the nuclei of replicate flasks at daily intervals by the method of Sanford *et al.*,[4] or by the production of colonies from single cells as described by Puck *et al.*[5] DNA and RNA syntheses were determined by the incorporation of P^{32}, H^3-thymidine, or H^3-cytidine. The incorporation periods were of 6-hour duration and were carried out in sequential 6-hour periods following irradiation. P^{32} incorporation was determined by isolating the nucleic acids (according to the method of Schmidt and Thannhauser[6]) and measuring their specific activities. The incorporation of H^3-thymidine into DNA was measured autoradiographically, the percentage of labeled cells in a total of approximately 8000 cells being counted for each interval. The number of cells incorporating H^3-cytidine into RNA was also measured by autoradiography after removing DNA by incubation with deoxyribonuclease.[7] The number of mitotic figures in a total of 10,000 to 40,000 cells in each group was determined by using fixed and stained preparations of cells grown on coverslips. The mitotic index has been expressed as the number of mitotic figures per 1000 cells. Radiation (220 kv, 15 mAmp., filtered through 0.5 mm. Cu and 1 mm. Al) was delivered at the rate of 100 to 126 r/min., and was usually administered on the second or third day after subculture.

Results and Discussion

Effect of radiation on cell replication. In untreated cultures the average doubling time of the cells was approximately 30 hours. The multiplication was reduced by irradiation, the extent of the inhibition being dependent on the dose.

The administration of 500 r almost completely inhibited further multiplication as evidenced by either nuclear counts or production of colonies (TABLES 1 and 2; FIGURE 1).

Effect of radiation on nucleic acid synthesis. Whether measured by specific activity of the isolated DNA or by the number of cells incorporating H^3-thy-

TABLE 1
EFFECT OF IRRADIATION ON MULTIPLICATION OF U-12 CELLS

Irradiation dose (r)	Total cells/flask* (% control)
100	60 (1)‡
125	46 ± 10† (2)
250	42 ± 12 (7)
375	21 ± 1 (2)
500	25 ± 9 (9)

* Cells were counted in 3 replicate flasks by the method of Sanford *et al.*[4] on the day 6 to 7 after inoculation or day 4 to 5 after irradiation.
† Average deviation
‡ Number of experiments.

TABLE 2
EFFECT OF IRRADIATION ON PRODUCTION OF COLONIES BY U-12 CELLS

Radiation dose (r)	No. colonies, (% control)
0	100 ± 11.1* (16)†
50	96.1 ± 19.8 (6)
106	80 ± 12.6 (8)
125	80.4 ± 11.0 (10)
175	65.5 ± 11.3 (6)
211	51.8 ± 18.5 (3)
250	41.0 ± 9.2 (8)
300	41.0 ± 9.2 (6)
319	43.1 ± 5.4 (4)
375	17.3 ± 6.6 (10)
500	2.92 ± 2.96 (4)

Two hundred cells were plated on a feeder layer previously irradiated with 2000 r. The newly plated cells were then irradiated as indicated, and visible colonies were counted 18 days later.
* Standard deviation.
† Number of flasks.

midine into DNA, the synthesis of DNA was found to be reduced only slightly in the first 6 hours following irradiation, but decreased slowly thereafter. At 18 to 24 hours following irradiation, the specific activity of the DNA, as well as the number of cells incorporating H^3-thymidine into DNA, was reduced to less than one half of the control values (FIGURE 2, curves B and C). The specific activity of the DNA is a measure of the rate of synthesis of DNA by the culture as a whole, which in turn depends on both the rate of synthesis in individual cells as well as the relative number of synthesizing cells. The relative number of synthesizing cells can be determined independently by autoradiography.

Since both methods of assay gave similar results (curves B and C in FIGURE 2), it is possible that the reduction in specific activity was entirely due to a reduction in the number of cells synthesizing DNA.

In contrast to the inhibition of DNA synthesis, there was no reduction in the specific activity of RNA nor in the number of cells incorporating H^3-cytidine into RNA at any of the time periods studied (TABLE 3). In control cultures, it was observed that after a 6-hour incorporation period, 97 to 100 per cent of

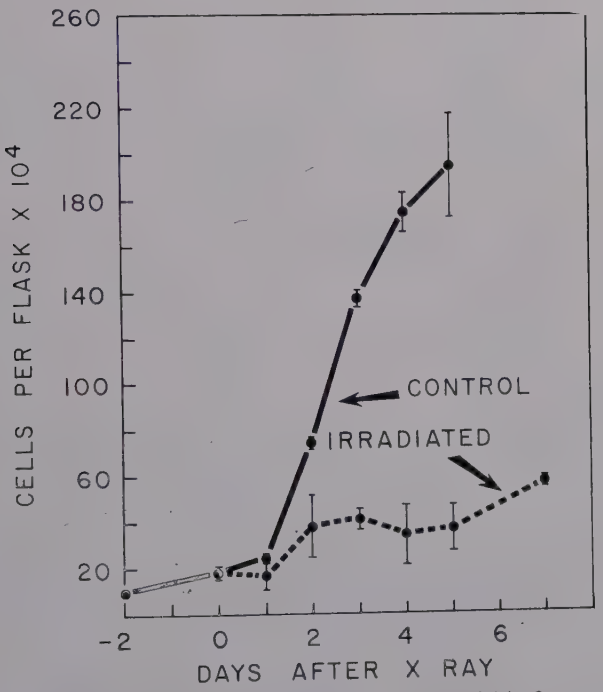


FIGURE 1. The effect of 500 r on cell replication. The nuclei in 3 control and 3 irradiated flasks were counted at daily intervals according to the method of Sanford *et al.*[4]

the cells had incorporated H^3-cytidine into RNA, while 47 per cent of the cells incorporated H^3-thymidine into DNA.

Effect of irradiation on mitosis. In contrast to the delayed effect on DNA synthesis, mitosis was inhibited almost immediately, the mitotic index falling to 5 per cent of the control value 105 min. after irradiation. Mitotic activity was not apparent for 12 hours; the dividing cells then again appeared and increased in frequency. As may be seen in curve A, FIGURE 2, the mitotic index was considerably above the normal value 18 to 20 hours after irradiation. Since the rise in mitotic index was not paralleled by an increase in cell number (curves A and D, FIGURE 2), the effect of irradiation on the mitotic process was studied by means of time-lapse photography. Observation of films showed that cells entering mitosis 15 to 20 hours after irradiation remained in mitosis for very long periods but seldom were successful in completing this process.

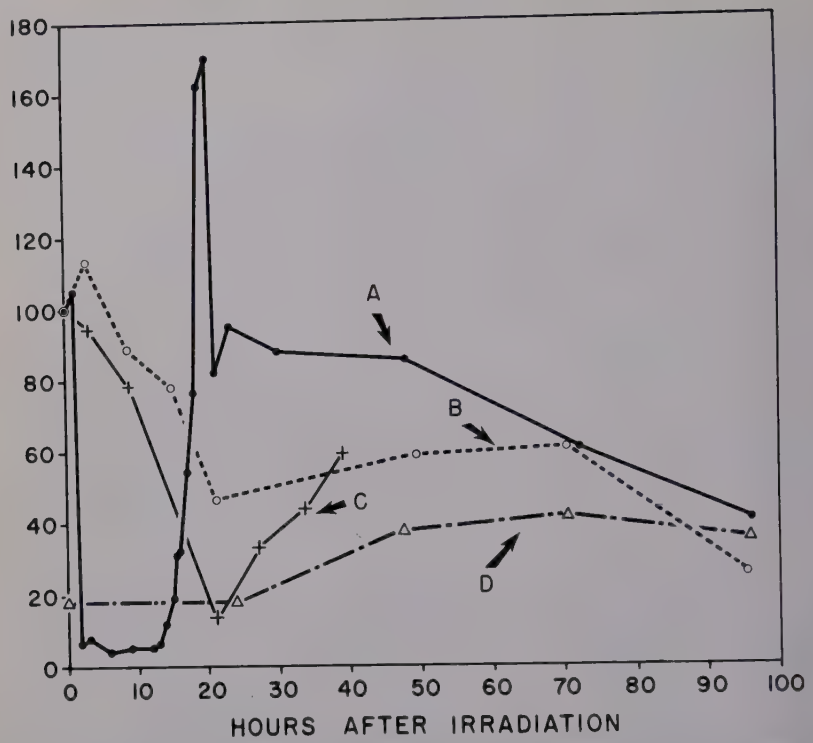


FIGURE 2. The effect of 500 r on cell division and DNA synthesis. Curve A, the mitotic index as percentage of the control value; curve B, the specific activity of DNA after a 6-hour incorporation of P^{32} expressed as percentage of the control value; curve C, the number of cells incorporating H^3-thymidine into DNA after a six-hour incorporation period, expressed as percentage of the control value; curve D, total cells per flask $\times 10^4$ as determined by the nuclear counting procedure of Sanford *et al.*[4]

TABLE 3
EFFECT OF 500 R ON RNA SYNTHESIS BY U-12 CELLS

Hours after 500 r	P^{32} incorporation into RNA specific activity (% control)	No. of cells incorporating H^3-cytidine into nuclear RNA (% control)
0–6	97 ± 16* (4)†	102 ± 1* (2)†
6–12	125 ± 18 (3)	103 ± 1 (2)
12–18	100 ± 5 (2)	101 ± 0 (2)
18–24	83 ± 14 (2)	102 ± 1 (2)
24–30		101 ± 1 (2)
30–36		102 ± 3 (2)
36–42		103 ± 1 (2)

P^{32} incorporation was measured by isolation of RNA from the harvested cells and determination of its specific activity. The number of cells incorporating H^3-cytidine into nuclear RNA was determined by autoradiography, treating the fixed cells with deoxyribonuclease.

* Average deviation.

† Number in parentheses refers to the number of experiments (P^{32}) or tubes (H^3-cytidine) per group.

Often a multinuclear giant cell was the result of the attempt. In control cultures, the average duration of mitosis was 64.1 min., while 15 to 70 hours after 500 r the average was 172.2 min. Of 32 attempted mitoses that were observed, only 3 were successful, whereas 29 were abortive.* In preparations of cells fixed and stained 18 hours after 500 r, the frequency of multinuclear cells was nearly 3 times that seen in control preparations. The abnormal nature of the mitoses occurring 15 or more hours after irradiation was also indicated by a comparison of the effects of colchicine and irradiation, alone and in combination. It may be seen in TABLE 4 that the addition of either colchicine or irradiation resulted in an increase in the mitotic index 20 hours after treatment, but that the combination of the two agents did not produce an additive effect. Therefore, both presumably acted by a similar mechanism; that is, delaying cells in the process of division. Prolonged and abortive mitoses occurring several hours after irradiation were first observed in tissue culture by

TABLE 4

EFFECT OF IRRADIATION AND COLCHICINE ON THE MITOTIC INDEX OF U-12 CELLS

Treatment	Mitotic index
Control	22.8 ± 3.1
Colchicine, 2×10^{-6} *M*, 20 hr.	48.7 ± 7.8
500 r, 20 hr. postirradiation	36.6 ± 3.1
Colchicine + 500 r, 20 hr. postirradiation	27.7 ± 4.5

The cells were grown in Leighton tubes. After irradiation the medium was changed in all tubes, and colchicine was added to those specified. Each group consisted of 4 tubes. A total of 7500 cells were counted per tube (a total of 30,000 per group). Mitotic index = mitotic figures per 1000 cells.

Strangeways in 1924,[8] and have been described in detail by Lasnitzki,[9] Simon-Reuss and Spear,[10] Brues and Stroud,[11,12] Pomerat,[13] and Boll.[14]

Effect of irradiation on the phases of the mitotic cycle. The division of the cell cycle into the phases G, S, G_2, and M was originally suggested by Howard and Pelc.[15] These phases were determined for the U-12 cells as described previously,[1] and are shown in FIGURE 3. Consideration of the cell cycle facilitated the interpretation of the results thus far described. Since mitosis was inhibited almost immediately after irradiation, cells in the G_2 interphase (occurring between DNA synthesis and mitosis) must have been prevented from entering prophase. Since cells in the G_2 phase already contain a double complement of DNA, a lack of DNA could not be the cause of mitotic inhibition occurring immediately after irradiation, as was pointed out by Howard in 1956.[16] On the other hand, the inhibition of mitosis might be expected to interfere with the normal progress of cells through the cycle and result in a reduction in the number of cells entering the phase of DNA synthesis (S) 17 hours after irradiation. The time course of the events that have been observed to occur in U-12 cells is in accord with this interpretation. Other studies of nucleic acid synthesis in irradiated tissue culture systems have also

* Time-lapse photography and preliminary analysis of the films were carried out by R. B. Adams of this department.

indicated that an inhibition of DNA synthesis was not an immediate effect, and perhaps occurred as a result of inhibited mitoses.[14,17-21]

In contrast to DNA synthesis, which occurs at a certain stage of the cell cycle,[15] cells apparently synthesize RNA at all stages except during actual

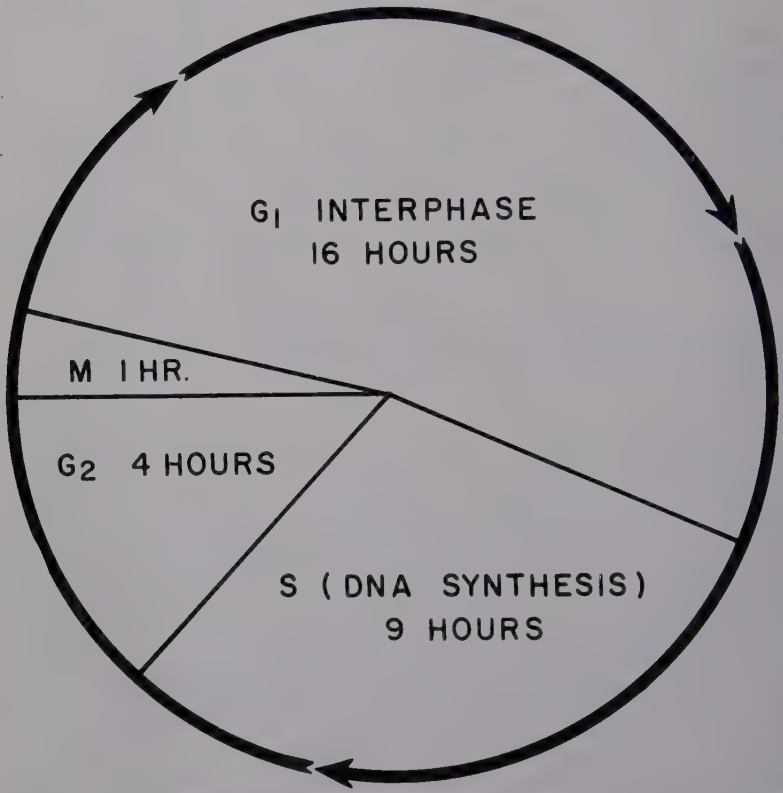


FIGURE 3. The mitotic cycle of strain U-12 fibroblasts. The mitotic time (M) was determined from the mitotic index and by direct timing with time-lapse photography. The length of G_2 was determined by the time necessary for the appearance of the first labeled mitotic figure after addition of H^3-thymidine. The length of the S period was calculated by the method of Painter and Robertson.[19] The doubling time of 30 hours was found by calculating the growth constant from the daily increase in the number of cells. The length of G_1 was calculated by difference. Reproduced from H. Harrington by permission of *Biochimica et Biophysica Acta*.[1]

mitosis.[22] Thus an interference with the progress of the cycle would not be expected to change the number of cells synthesizing RNA to a significant extent. Or, as stated by Howard,[16] since DNA synthesis is uniquely dependent on the mitotic cycle, "DNA synthesis might be expected to be unique in its response to radiation-induced changes in that cycle."

The discrepancy between the rise in mitotic index and the absence of actual cell multiplication (curves A and D, FIGURE 2) can be explained by the large preponderance of abortive divisions, many giving rise to multinuclear cells.

According to Carlson,[23] a rise in the mitotic index could be caused by an increase in the number of cells entering mitosis, a decrease in the cells leaving mitosis, or both. Observations made with time-lapse photography have shown that the rise in mitotic index occurring 18 to 20 hours following the irradiation of U-12 cells was due at least in part to a decrease in cells leaving mitosis. However, it seemed possible that an increased number of cells might also be entering mitosis at certain times after irradiation. Such an increase could result from the simultaneous entry into mitosis of cells that have been delayed (—in G_2—) and cells which were in an earlier part of the cycle (—G_1—) at the time of irradiation and have had time to recover before reaching mitosis.[24] The number of U-12 cells entering mitosis at various times after irradiation and the stage of these cells in the mitotic cycle at the time of irradiation were therefore investigated.

Twelve hours after irradiation, the medium of irradiated and control tubes was changed to medium containing colchicine and H^3-thymidine. Colchicine was added in order to arrest the mitoses occurring in the controls so that they might be compared to the irradiated mitoses. At 2-hour intervals after H^3-thymidine addition, the cells were fixed and autoradiographs prepared. The number of labeled and unlabeled mitotic figures was recorded and the mitotic index determined. The results are shown in FIGURE 4. In part A it can be seen that there were more irradiated cells entering mitosis 16–20 hours after irradiation, or 4 to 8 hours after colchicine and H^3-thymidine addition, than there were unirradiated cells. In the case of both irradiated and controls, some of the cells entering mitosis 2 to 10 hours after addition of H^3-thymidine (14 to 22 hours after irradiation) were labeled (B, FIGURE 4). Since the irradiated cells were able to incorporate the H^3-thymidine into DNA and move through G_2 into mitosis at a rate similar to, or slightly faster than, the controls, it is concluded that cells passing through the G_2 phase 12 or more hours after 500 r were not delayed.

Many more unlabeled mitotic figures were found in the case of the irradiated than control cells (C, FIGURE 4). These unlabeled cells must have been in the G_2 period at the time of H^3-thymidine addition and therefore must have accumulated in this phase in the first 12 hours following the irradiation. These data indicate, then, that 12 hours after irradiation the delay in the G_2 phase had disappeared, and that the cells that entered mitosis after this time consisted of cells which had previously accumulated in the G_2 phase as well as cells coming from the synthetic phase.

Additional characterization of cells appearing in mitosis 12 or more hours after irradiation was carried out using sequential 6-hour labeling periods with H^3-thymidine. These periods consisted of (1) the 6 hours preceding irradiation; (2) the first 6 hours following irradiation; (3) 6 to 12 hours following irradiation; and (4) 12 to 18 hours following irradiation. After the 6-hour incorporation period, the cells were washed in medium containing 0.05 μM of nonradioactive thymidine per ml. and were then incubated in this medium until they were fixed at 18 hours after irradiation. The relative number of labeled and unlabeled mitotic figures was then determined; the results are shown in TABLE 5. The only cells that could give rise to unlabeled mitotic figures 18 hours after irradiation are indicated for each labeling period. With the first labeling

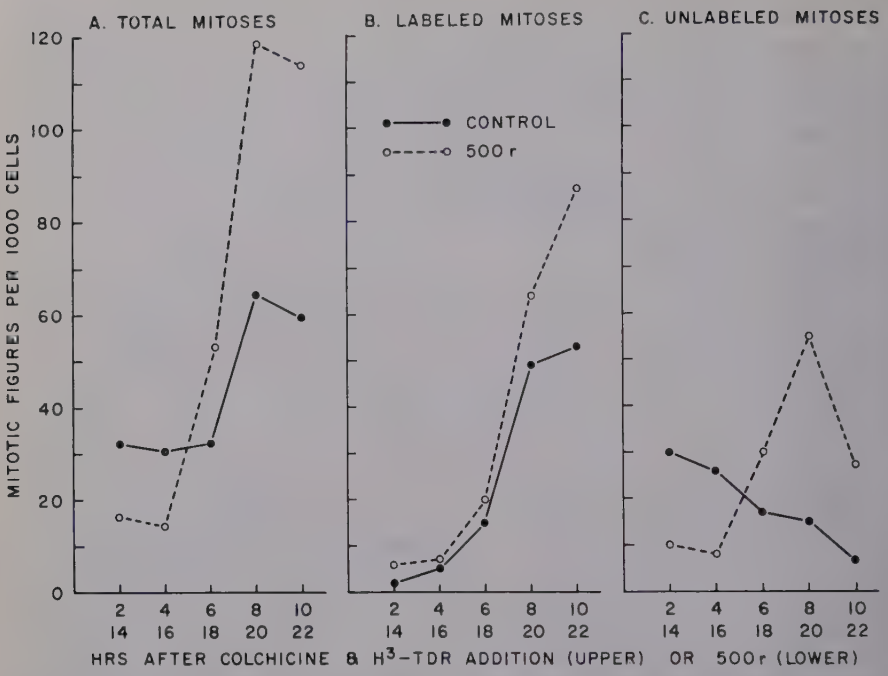


FIGURE 4. Accumulation of U-12 cells in mitosis after irradiation and/or colchicine. Twelve hours after 500 r, the medium was changed to one containing colchicine (2×10^{-5} *M*) and H^3-thymidine (0.05 μc./0.01 μM/ml.). Cells were fixed at 2-hour intervals and autoradiographs prepared. The number of mitotic figures was counted in a total of 30,000 to 40,000 cells for each interval, and the mitotic index (mitotic figures/1000 cells) calculated. A total of 600 to 800 mitotic figures was observed at each interval for the presence or absence of labeling.

TABLE 5

APPEARANCE OF NONLABELED U-12 CELLS IN MITOSIS 18 HOURS AFTER 500 R AND VARIOUS LABELING PERIODS WITH TRITIATED THYMIDINE

Labeling period	History of nonlabeled cells appearing in mitosis	Nonlabeled mitotic figures (%)
(1) Six hours preceding X ray	G_1 at time of X ray	40 ± 4*
(2) Zero to 6 hours after X ray	G_2 at time of X ray	27 ± 5
(3) Six to 12 hours after X ray	G_2 at time of X ray and any cells in S at the time of X ray that accumulated in G_2 in the first 6 hours after X ray	21 ± 6
(4) Twelve to 18 hours after X ray	G_2 at time of X ray and any cells in S or G_1 at the time of X ray that accumulated in G_2 in the first 12 hours after X ray	35 ± 6

* Average deviation between tubes. Three to four tubes were counted per group, the total mitotic figures observed ranging from 454 to 1293.

Concentration of thymidine was 0.05 μc./0.01 μM/ml.

period, the only cells that could give rise to unlabeled mitotic figures 18 hours after irradiation were cells which were in the G_1 phase at the time of irradiation. Since 40 per cent of the mitotic figures were unlabeled after this treatment, it is indicated that some of the cells appearing in mitosis consisted of cells which had been in the G_1 interphase at the time of irradiation. Similarly, with the 0- to 6-hour labeling period, only cells which were in the G_2 phase at the time of irradiation could produce unlabeled mitotic figures 18 hours later. Since 27 per cent of the cells in mitosis were nonlabeled after this treatment, cells in the G_2 phase at the time of irradiation also contributed to the observed mitotic figures. When the labeling period was 6 to 12 hours after irradiation, cells that were in the G_2 phase at the time of irradiation and any cells that had accumulated in the G_2 phase before H^3-thymidine addition could give rise to unlabeled mitotic figures 18 hours after irradiation. Since the percentage of unlabeled mitotic figures was no higher with this labeling period than in the second period (0 to 6 hours after irradiation) it is indicated that no cells moved from the synthetic phase into the G_2 phase in the first 6 hours following irradiation. This result is in agreement with that of Painter and Robertson, who found that HeLa cells were retained in the synthetic period for the first 8 hours following 500 r.[19] With the fourth labeling period of 12 to 18 hours postirradiation, only those cells that were in the G_2 phase at the time of irradiation and those which accumulated in the G_2 phase during the first 12 hours following irradiation would give rise to unlabeled mitotic cells. Since a higher percentage of unlabeled mitoses were found in this group than in group 2 or 3, it is indicated that cells from the G_1 or S phases do accumulate in the G_2 period between 6 and 12 hours following irradiation. The results of this experiment are in agreement with those shown in FIGURE 4 in indicating that the cells appearing in mitosis 18 hours after irradiation consist of cells which were in the G_2, S, and G_1 phases at the time of irradiation. These conclusions have been based on the temporal relationships of the phases in the normal mitotic cycle (FIGURE 3) and the assumption that irradiation affects the cycle only by prolonging various phases. Apparently during the first 6 hours following irradiation the cells are retained in both G_2 and S. A G_1 retention evidently does not occur in HeLa cells irradiated with 500 r.[19] However, Lajtha *et al.* concluded that myelocytes grown in culture were delayed in the G_1 period by 135–500 r.[25] Preliminary data have indicated that U-12 cells are not retained in the G_1 period,[26] but more experiments are necessary to confirm this observation. The data in FIGURE 4 and TABLE 5 indicate that sometime after 6 hours after irradiation cells move out of the synthetic phase and accumulate in G_2. At about 12 hours the block in the G_2 period seems to disappear and cells which have accumulated in G_2, as well as those passing from S through G_2, start mitosis but are unable to complete it normally. The reduction in the number of cells synthesizing DNA 18 to 24 hours after irradiation could be explained by the block in the cycle at mitosis and subsequent depletion of the G_1 and S periods.

Summary

The effect of irradiation on Strain U-12 fibroblasts has been studied. The administration of 500 r inhibited mitosis almost immediately, whereas the

specific activity of DNA and the number of cells synthesizing DNA were not reduced markedly until 18 to 24 hours following the irradiation. RNA synthesis was not reduced at any interval observed. Twelve hours after irradiation mitotic figures again appeared and increased in frequency to above the normal level 18 to 20 hours after irradiation. The increase in mitotic index was found to be caused both by an increase in the cells entering and by a decrease in cells leaving mitosis. The cells entering mitosis 12 or more hours after irradiation appeared to consist of cells that had accumulated in the G_2 period 6 to 12 hours after irradiation and cells coming from the synthetic period that were able to pass through the G_2 period without delay 12 hours after irradiation. Cells entering mitosis 12 or more hours after irradiation were found to spend long periods trying to divide but were most often unsuccessful and, in many cases, they produced a multinuclear giant instead of two normal daughter cells.

References

1. Harrington, H. 1960. Biochim. Biophys. Acta. **41:** 461.
2. Swim, H. E. & R. F. Parker. 1957. Am. J. Hyg. **66:** 235.
3. Swim, H. E. & R. F. Parker. 1958. J. Lab. Clin. Med. **52:** 309.
4. Sanford, K. K., W. R. Earle, V. J. Evans, H. K. Waltz & J. E. Shannon. 1951. J. Nat. Cancer Inst. **11:** 773.
5. Puck, T. T., P. I. Marcus & S. J. Cieciura. 1956. J. Exp. Med. **103:** 273.
6. Schmidt, G. & S. J. Thannhauser. 1945. J. Biol. Chem. **161:** 83.
7. Moses, M. J. & J. H. Taylor. 1955. Exp. Cell Research. **9:** 474.
8. Strangeways, T. S. P. 1924. Proc. Roy. Soc. B. **96:** 291.
9. Lasnitzki, I. 1940. Brit. J. Radiol. **13:** 279.
10. Simon-Reuss, J. & F. G. Spear. 1947. Brit. J. Radiol. **20:** 63.
11. Brues, A. M., A. N. Stroud & G. Svihla. 1951. Anat. Record. **109:** 411.
12. Stroud, A. N. 1956. Ann. N. Y. Acad. Sci. **67:** 11.
13. Pomerat, C. M., S. P. Kent & L. C. Logie. 1957. Z. für Zellforsch. **47:** 175.
14. Boll, I. 1958. Haematol. Latina (Milan). **1:** 172.
15. Howard, A. & S. R. Pelc. 1953. Heredity, Suppl. **6:** 261.
16. Howard, A. 1956. *In* Ciba Foundation Symposium on Ionizing Radiations and Cell Metabolism. : 196. B. E. W. Wolstenholme and C. M. O'Connor, Eds. Little, Brown. Boston, Mass.
17. Whitmore, G. F., J. E. Till, R. B. L. Gwatkin, L. Simonovitch & A. F. Graham. 1958. Biochim. Biophys. Acta. **30:** 583.
18. Dickson, M., J. Paul & J. N. Davidson. 1958. Biochem. J. **70:** 18P.
19. Painter, R. B. & J. S. Robertson. 1959. Radiation Research. **11:** 206.
20. Whitfield, J. F. & R. H. Rixon. 1959. Exp. Cell Research. **18:** 126.
21. Till, J. E. 1961. Use of Animal Cell, Tissue, and Organ Cultures in Radiobiology. Ann. N. Y. Acad. Sci. **95**(2): 911.
22. Feinendegen, L. E., V. P. Bond, W. W. Shreeve & R. B. Painter. 1960. Exp. Cell Research. **19:** 443.
23. Carlson, J. G. 1954. *In* Radiation Biology. **1:** 767. A. Hollaender, Ed. McGraw-Hill. New York, N. Y.
24. Canti, R. G. & F. G. Spear. 1929. Proc. Roy. Soc. B. **105:** 93.
25. Lajtha, L. G., R. Oliver, T. Kumatori & F. Ellis. 1958. Radiation Research. **8:** 1.
26. Harrington, H., E. M. Seidman & P. S. Lavik. 1959. Federation Proc. **18:** 242.

RADIATION EFFECTS ON THE DIVISION CYCLE OF MAMMALIAN CELLS *IN VITRO**

J. E. Till

Department of Medical Biophysics, University of Toronto, and the Physics Division, the Ontario Cancer Institute, Toronto, Ont., Canada

It is now well established that deoxyribonucleic acid (DNA) synthesis, like mitosis, is an event that occupies only a portion of the division cycle in mammalian cells.[1] The regularly repeated time interval between DNA synthesis and mitosis implies the existence of a coupling between these processes. If there is such a coupling, inhibition of one process by an agent such as ionizing radiation might be expected to affect the other. This has been shown to be the case for cells that have suffered a radiation-induced delay of mitosis; the subsequent synthesis of DNA is delayed to a similar extent.[2,3] It is of considerable interest to know whether the reciprocal process occurs, that is, whether any *specific* effect on DNA synthesis *precedes* the inhibition of mitosis by ionizing radiation.[4]

The Division Cycle of L Cells

The time relationship between DNA synthesis and mitosis for exponentially multiplying strain-L mouse cells in suspension culture is shown diagrammatically in FIGURE 1. The approximate durations of the DNA synthesis (S) period, the pre-DNA synthesis (G1) period, the post-DNA synthesis (G2) period, and mitosis (M) are indicated. The approximate percentage of cells in the population that are in each period at any time is also given. The methods used to obtain these results have already been published.[5,6]

Inhibition of Cell Division in Irradiated Cultures

This paper will be concerned with the effects of radiation on the periods of the division cycle shown in FIGURE 1, during the *first cycle following irradiation.* Suspension cultures of L cells are particularly useful for this type of study. The materials and methods used in the experiments to be discussed below have already been described.[3]

One effect that becomes apparent shortly after irradiation is radiation-induced mitotic delay, or "preprophase arrest," which results in a rapid decrease in the number of visible mitotic figures.[2,3,7] The occurrence of mitotic delay in mammalian cells is in agreement with results obtained with other materials, which indicate that the retardation of mitosis occurs at a stage just previous to that recognized as prophase.[8] The duration of mitotic delay depends on radiation dose in the manner illustrated in FIGURE 2. Over the dose range shown in the figure, the mitotic delay is linearly related to the logarithm of the dose. It may be noted that following a radiation dose of 2000 r, the delay is 13 hours, while this time is increased to 20 hours following a dose of 5000 r.

* The work described in this paper was supported in part by a grant from the National Cancer Institute of Canada, Toronto, Ont., Canada.

Although mitotic figures reappear in the cultures following such radiation doses, this does not necessarily mean that the proliferative capacity of the cells has been restored, because a large majority of the cell divisions that occur are abortive, in the sense that the cells may undergo one or two divisions, but they are incapable of continued proliferation. This is indicated by the data of FIGURE 3, which shows the survival curve of colony-forming ability. For a

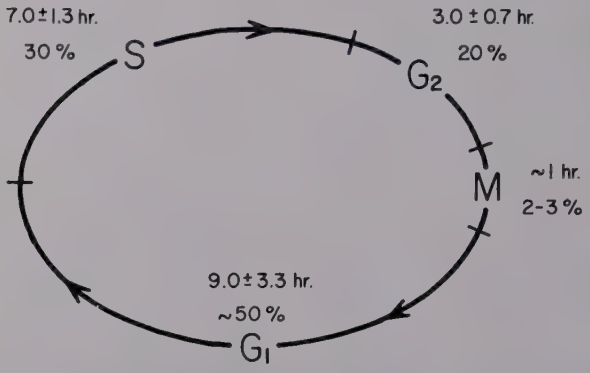


FIGURE 1. The division cycle for exponentially multiplying strain-L mouse cells in suspension culture (see text).

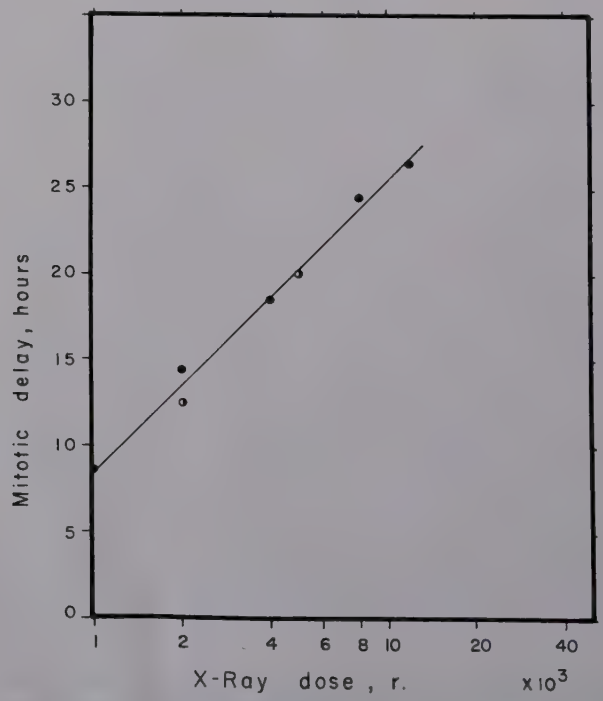


FIGURE 2. The duration of mitotic delay as a function of X-ray dose (logarithmic scale).

dose of 2000 r, only about 0.1 per cent of the cells are capable of carrying out a number of divisions sufficient to form a macroscopic colony. Thus a dose as large as 2000 r has a very profound effect on cell proliferation. The reappearance of mitotic figures is delayed for 13 hours, that is, more than one-half a generation time following irradiation, and only one cell in a thousand retains a capacity for continued proliferation.

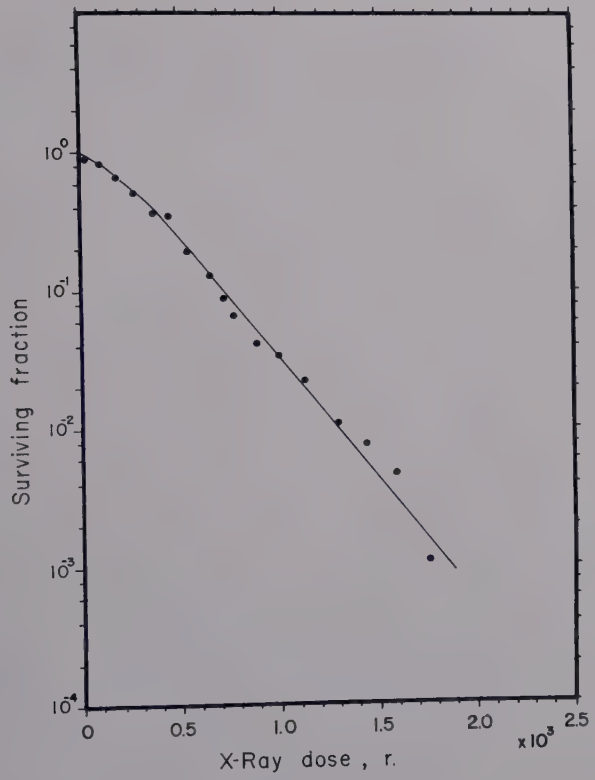


FIGURE 3. Survival curve for the colony-forming ability of L cells as a function of X-ray dose.

The Rate of DNA Synthesis at Early Times After Irradiation

To gain some information concerning the rates of DNA synthesis in individual cells, a 5000 r-irradiated culture and an unirradiated control culture were exposed to tritium-labeled thymidine (H^3TDR) for a 1-hour period. Autoradiographs of the cells from these cultures were obtained, and counts were made of the number of developed grains appearing in the photographic emulsion over labeled cells. FIGURE 4 illustrates histograms of the frequency distribution of these grain counts for the control and the 5000 r irradiated cultures. These populations are both heterogeneous with respect to grain numbers, but statistical analysis indicates that there is no significant difference in the form of the

frequency distribution of grain numbers for the control and the irradiated cell populations. This finding, coupled with the observation that the percentage of labeled cells was very nearly the same in both populations, indicates that there is a decrease in the rate of DNA synthesis in the individual cells in the irradiated culture; that is, irradiation has not stopped certain cells from synthesising DNA while allowing others to continue. All cells in the S period appear to have been affected to some extent.

Dividing the mean grain count over labeled irradiated cells by the mean grain count over labeled unirradiated cells gives a value that represents the depression in the rate of DNA synthesis due to irradiation. Values obtained

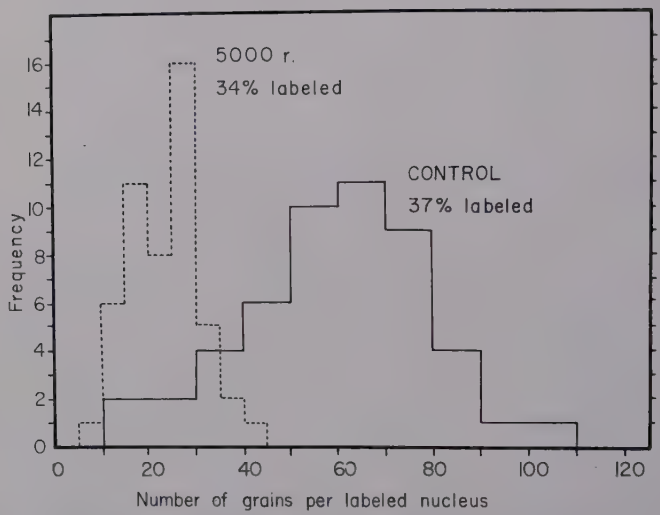


FIGURE 4. Frequency distributions of grain counts over labeled nuclei in autoradiographs prepared from control and 5000 r-irradiated cultures exposed to H^3-thymidine for 1 hour, 100 min. after irradiation. Control culture: per cent labeled cells, 37; mean grain count per labeled nucleus, 59. Irradiated culture: per cent labeled cells, 34; mean grain count per labeled nucleus, 24. Ratio, irradiated to control grain counts, 0.41. (Data of S. Mak.)

at various times after irradiation are listed in TABLE 1. At a dose level of 2000 r, which strongly inhibits mitosis, the rate of DNA synthesis immediately after irradiation is only slightly affected. For at least the first 3 hours after irradiation, DNA synthesis occurs at about 80 per cent of the rate shown by the controls. For a much higher dose of 5000 r, the rate of DNA synthesis is decreased to about 50 per cent of the control rate. It will be shown however that this decrease is not entirely due to a *specific* depression of DNA synthesis but is rather, at least in part, the result of a generalized slowing down of the movement of cells around the division cycle.

It is worthy of note that the effect of radiation on the rate of DNA synthesis does not depend on whether the cell has to perform *de novo* synthesis of deoxynucleosides (other than thymidine), since the measured depression in the rate of synthesis after irradiation measured by H^3TDR uptake is the same in the presence or absence of added deoxynucleosides (TABLE 1).

As a check on the reliability of H^3TDR labeling as a criterion of DNA synthesis, the increase in the total DNA of the culture as a function of time after irradiation was measured colorimetrically.[3] The results are shown in FIGURE 5. The results are based on an initial cell population of 10^5 cells per milliliter. In the control culture the increase in DNA is due to cell multiplication, with a doubling time of close to 20 hours. In the irradiated cultures the number of cells present did not change over the first 15 hours after irradiation; thus the increase in DNA is due to an increase in DNA per cell.[3] It is apparent that the initial rate of increase in DNA in the irradiated cultures is slightly depressed relative to the control culture. The rate of synthesis is about 85 per cent of the control rate in the 2000 r culture and about 60 per cent of the control rate in the 5000 r culture, so that the depression in the rate of increase of total DNA is similar

TABLE 1

THE RATE OF UPTAKE OF H^3-THYMIDINE BY IRRADIATED L CELLS*†

Time of addition of H^3TDR following irradiation (min.)	H^3 activity in labeled irradiated cells / H^3 activity in labeled control cells			
	2000 r‡	5000 r‡ Expt. 1	5000 r‡ Expt. 2	5000 r§ Expt. 3
10	0.78	0.45	0.49	—
70	0.78	0.43	0.59	0.43
130	0.88	0.47	0.48	0.56
190	0.74	0.37	0.49	0.54
Mean	0.80 ± 0.06	0.43 ± 0.04	0.51 ± 0.05	0.51 ± 0.08

* Data of S. Mak.

† One-hour exposure to label.

‡ Growth medium 1066[9] containing deoxynucleosides (thymidine, deoxymethylcytidine, deoxycytidine, deoxyadenosine, deoxyguanosine) at a concentration of 10 μg./ml.

§ Growth medium 1066 without deoxynucleosides.

to the depression in rate of synthesis in individual cells, measured by means of H^3TDR incorporation.

The average DNA content per cell in an exponentially multiplying culture is 1.3 μg. per 10^5 cells,[3] while the premitotic DNA content of cells in the G2 period is 2.0 μg. per 10^5 cells.[5] Since the average DNA content of cells in irradiated cultures approaches a value of 2.0 μg. per 10^5 cells within about 20 hours after irradiation (FIGURE 5), it may be assumed that most of the cells in the irradiated cultures are able to pass through the S period, attain the premitotic content of DNA, and enter the G2 period during the time that mitosis is delayed. If most of the cells in the irradiated cultures are able to complete the synthesis of DNA, as this implies, the decrease in the rate of DNA synthesis in individual cells in the irradiated cultures must be the reflection of a decrease in the rate of movement of cells through the S period.

Radiation Effects on the Movement of Cells from G1 into S

The data of TABLE 1 suggest that the moderate depression in the rate of DNA synthesis that occurs following irradiation is not confined to cells that were in

the S period at the time of irradiation. By 3 hours after irradiation, a significant portion of the cells in the S period should consist of cells that were in the G1 period at the time of irradiation, yet the rate of uptake of H^3TDR remained depressed. Since the decreased rate of DNA synthesis in individual cells appears to be a reflection of a decreased rate of movement through the S period, the question arises as to whether the decreased rate of movement following irradiation is confined to the S period, or whether it extends into the G1 period, so that the rate of entry of cells into the S period is also depressed. This may be determined by examining the rate of increase of labeled cells taking place in the continued presence of H^3TDR.[3] The results of such an experiment are

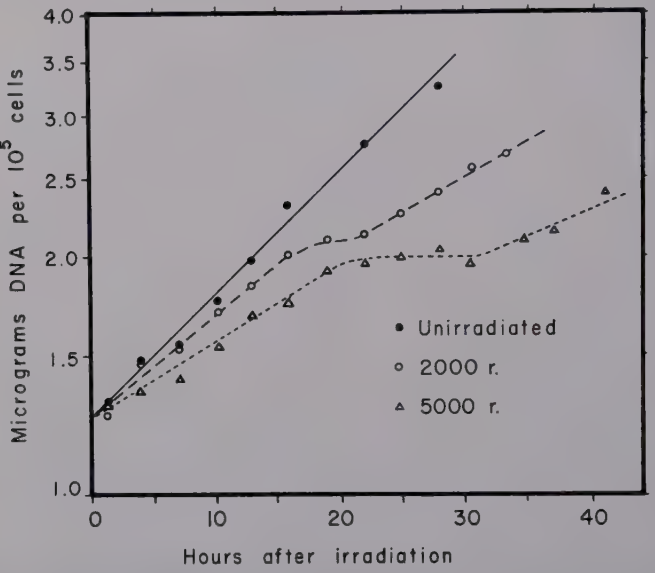


FIGURE 5. The DNA content of control, 2000 r-irradiated, and 5000 r-irradiated cultures as a function of time after irradiation. The units on the ordinate scale represent micrograms of DNA per 10^5 cells initially present in the cultures at the time of irradiation (see text).

shown in FIGURE 6. The percentage of labeled cells in the unirradiated culture increases with time, reaching a value very close to 100 per cent within 13 hours after addition of H^3TDR. This is the time required for cells to traverse the G2, M, and G1 periods, that is, to proceed from the end of the previous S period to the beginning of the next. In cultures exposed to radiation, the percentage of labeled cells is initially the same as in the control, that is 30 per cent, and thereafter increases at a slightly reduced rate, levelling off temporarily at 80 per cent. The cells that fail to incorporate label on schedule are thought to be the 20 per cent of the cells in the population that are trapped in the G2 period by the preprophase radiation-induced block that is responsible for mitotic delay.[3] The data of FIGURE 6 indicate that cells exposed to 2000 r leave the G1 period and enter S at 90 per cent of the control rate, while cells exposed to 5000 r enter the S period at about 70 per cent of the control rate. Thus the depres-

sion by radiation of the rate of movement of cells from G1 into S is almost the same as the depression of the rate of movement through S.

The fact that the number of cells initially taking up label is the same in both the control and the irradiated cultures (FIGURE 6) provides support for the view that most of the cells in the irradiated cultures are still capable of movement through the S period immediately following irradiation. The observation that a total of 80 per cent of the cells in the irradiated cultures are able to take up label within 10 to 13 hours after irradiation indicates that most of the cells in G1 are able to pass through G1 and enter the S period during the time that mitosis is delayed in the cultures.

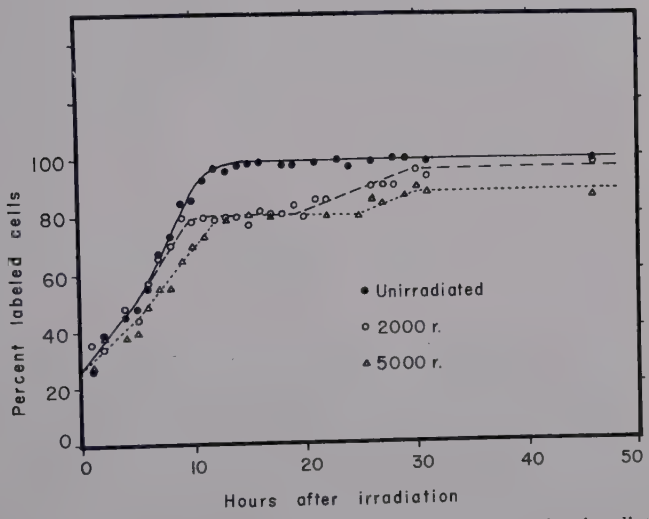


FIGURE 6. The percentage of labeled cells as a function of time after irradiation, in control, 2000 r-irradiated, and 5000 r-irradiated cultures. H^3-thymidine was added immediately following irradiation.

The Cell Cycle of Irradiated Cells

The results presented above indicate that there is little, if any, *specific* effect of radiation on the rate of movement of cells through the DNA synthesis period. The evidence bearing on this view is summarized diagrammatically in FIGURE 7. The values shown for the durations of the G1 and S periods in irradiated cells are based on the reduced rates of movement of irradiated cells through these periods, as indicated by the data of FIGURES 5 and 6, and TABLE 1. Although the average duration of the G1 and S periods is apparently increased by irradiation, they do not appear to suffer any serious distortion in the first hours following irradiation. A G2 period is, however, difficult to define in irradiated cells, since it appears that the orderly movement of cells through the G2 period is disrupted by radiation. If H^3TDR is added at the time of irradiation, the first cells appearing in mitosis following the period of mitotic delay induced by 5000 r normally contain label, indicating that cells that were in the G1 or S periods at the time of irradiation suffer considerably less delay

than cells that were in the G2 period (C. P. Stanners, unpublished observations). In FIGURE 7 the G2 period has been replaced by a period designated G2′, which has been set equal in duration to the period of mitotic delay.

It is apparent from FIGURE 7 that the changes induced in the division cycle by radiation are dependent on radiation dose, and the most profound effect at early times after irradiation is on cells that have completed DNA synthesis, that is, the cells in G2. In these cells movement out of the G2 period into mitosis quickly stops, and the reappearance of mitotic figures is markedly delayed.

The duration of mitosis following irradiation has not been studied in detail in these cells. Some cells probably fail to enter mitosis after irradiation, while others may spend an increased length of time in mitosis.[7,3]

Although the duration of the DNA synthesis period is increased by radiation, the increase is not specific, as the duration of the G1 period is also increased to

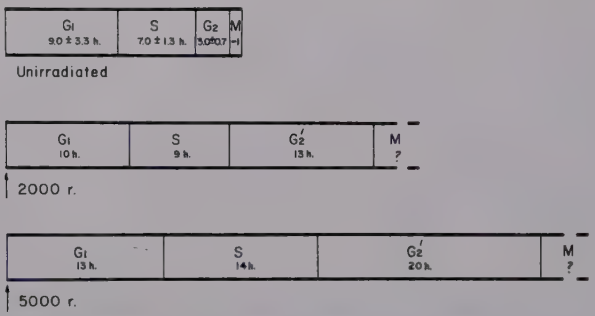


FIGURE 7. The durations of the periods of the division cycle of L cells in the first cycle following irradiation with 2000 r or 5000 r, compared to the division cycle of unirradiated L cells.

an almost equivalent extent. Thus exposure of mammalian cells to ionizing radiation appears to cause a generalized, dose-dependent increase in the duration of the known stages of the division cycle, with the greatest influence being on the post-DNA synthesis (G2) period immediately prior to mitosis.

The conclusion that may be drawn from these results is the following. In studying the effects of radiation on the division cycle of mammalian cells, it is necessary to distinguish between specific effects on a particular period such as the DNA synthesis period and general effects on all parts of the cycle. If any specific effect on DNA synthesis does precede the inhibition of mitosis by radiation, it would appear that improved techniques will be required to detect it with certainty in this system. Any pronounced effects of irradiation on DNA synthesis that have been observed in mammalian cells in tissue culture appear to be the result, rather than the cause, of inhibition of mitosis.

Acknowledgments

I am grateful to G. F. Whitmore, S. Mak, and C. P. Stanners for their assistance and advice.

References

1. Taylor, J. H. 1960. Nucleic acid synthesis in relation to the cell division cycle. Ann. N. Y. Acad. Sci. **90**(2): 409–421.
2. Harrington, H. 1960. Effect of irradiation on cell division and nucleic acid synthesis in strain U-12 fibroblasts. Biochim. Biophys. Acta. **41:** 461–469.
3. Whitmore, G. F., C. P. Stanners, J. E. Till & S. Gulyas. 1961. Nucleic acid synthesis and the division cycle in X-irradiated L-strain mouse cells. Biochim. Biophys. Acta. **47:** 66–77.
4. Hevesy, G. 1945. On the effect of roentgen rays on cellular division. Revs. Mod. Phys. **17:** 102–111.
5. Stanners, C. P. & J. E. Till. 1960. DNA synthesis in individual L-strain mouse cells. Biochim. Biophys. Acta. **37:** 406–419.
6. Stanners, C. P. 1960. An autoradiographic study of DNA synthesis in individual L-strain mouse cells. M.A. thesis. University of Toronto.
7. Harrington, H. 1961. The effect of X irradiation on the progress of strain U-12 fibroblasts through the mitotic cycle. Ann. N.Y. Acad. Sci. **95**(2): 901–910.
8. Lea, D. E. 1955. Actions of Radiations on Living Cells, 2nd ed. Cambridge University Press. New York, N.Y.
9. Parker, R. C., L. N. Castor & E. A. McCulloch. 1957. Altered cell strains in continuous culture: a general survey. *In* Cellular Biology, Nucleic Acids and Viruses. Spec. Publ. N. Y. Acad. Sci. **5:** 303–313.

IRRADIATION OF SELECTED PARTS OF SINGLE CELLS*

T. R. Munro
Strangeways Research Laboratory, Cambridge, England

Introduction

Since Vintemberger[1] studied the results of irradiating either the nucleus or most of the cytoplasm of frogs' eggs with X rays, a number of experiments on the relative radiosensitivity of nucleus and cytoplasm have been reported. In studies with ionizing radiations, if cell death were the criteria by which sensitivity was judged, the nucleus usually appeared the more sensitive. Most work of this kind has been done with biological material that was chosen either because some operation akin to nuclear transplantation could be carried out upon it, so that a normal nucleus could be implanted into irradiated cytoplasm or vice versa, or because the geometry of its cytoplasm and nucleus made it particularly favorable for localized irradiation.

Among experiments of the former type, Astaurov and Ostriakova-Varshaver[2] reported that silkworm eggs were killed by 30,000 r of X radiation, but could be made to develop androgenetically by fertilization with unirradiated sperm after doses as high as 540,000 r. An example of the latter type is provided by the elegant work of Rogers and von Borstel[3,4] who compared the effect of irradiating the nuclear region of the parasitic wasp Habrobracon with alpha particles, with the effect of irradiating most of the cytoplasmic surface. These authors found that if eggs were to be killed by the cytoplasmic irradiation, the dose required was some 3000 times as great as that needed when the nucleus was irradiated, and that death due to cytoplasmic irradiation occurred later in development. While it is probably true that the nucleus of these eggs is more radiosensitive than the cytoplasm, these experiments, like most of those in which microbeams or the limited range of monoenergetic alpha particles have been employed to permit localized irradiation, are open to the criticism that only a part of the cytoplasm was irradiated and that the rest might have taken over much of the function of the damaged part; the only kind of cytoplasmic damage that would almost certainly be revealed by such experiments, is a radiation-induced release of toxic substances. In the Habrobracon eggs the ratio between nuclear and cytoplasmic sensitivities would probably have been found to be smaller if the whole of the cytoplasm could have been irradiated.

While these experiments are of considerable interest, the extrapolation from insect eggs to the mammalian cells that are of most interest in radiobiology is a long one, and studies of the true relative radiosensitivity of nucleus and cytoplasm in animal cells grown in culture would be extremely desirable, particularly if cell survival were the criterion by which sensitivity were judged. Tissue culture cells are less favorable for this type of investigation than insect eggs, but in recent years several workers have devised culture methods and

* The work described in this paper was supported by the Prophit Trust of the Royal College of Surgeons, the Saltwell Trust of the Royal College of Physicians, and the British Empire Cancer Campaign, London, England. The experiments were performed in the Radiobiology Department, Christie Hospital and Holt Radium Institute, Manchester, England.

physical apparatus that allow localized irradiation to be achieved. The first half of this paper describes the results of short-term experiments in which parts of dividing cells in cultures from the hearts of 7-day chick embryos were irradiated with alpha irradiation, and examined for anomalies in anaphase and nuclear reconstruction at telophase. The results were suggestive, but such short-term experiments could not provide a direct answer to the question of whether it is the effect of the radiation upon the nucleus or the cytoplasm that deprives cells of reproductive integrity. The second half of the paper deals with tissue culture methods that both allow long term (for example, 7-day) studies to be made of single irradiated cells, and enable a large proportion of the cytoplasm of an intermitotic cell to be irradiated while the nucleus remains untouched.

Localized Irradiation of Single Cells in Culture

Methods. The technique that enabled parts of cells to be irradiated is based on the fact that polonium emits α particles that have a well-defined range of about 40 μ in water[5] or soft tissue of unit density. Very small sources were made by depositing polonium upon the tips of tungsten microneedles; the element was sealed in position by an electrodeposited layer of chromium.[6] The α particles lost some of their energy in passing through the chromium and emerged with a range that varied between 20 and 35 μ, but was still well defined for a given needle. The dose rate as a function of distance from the needle was measured by a microscintillation counting technique.[7] Cultures for irradiation were mounted over liquid paraffin on chambers similar to the chambre à huile of Commandon and de Fonbrune;[8] by placing one of the microneedles at an appropriate distance from a cell, a selected region at one end of it could be irradiated, for example the zone containing one group of daughter chromosomes in a cell at late anaphase (FIGURE 1), or part of the cytoplasm in a cell at metaphase.

The usual procedure was to select a cell, expose a few frames of 16 mm. film to provide a record of its appearance before irradiation, and to irradiate it by placing a microneedle at the required distance, usually for 1 min. Observation and filming were continued during and after irradiation; when the observations had been completed the culture was fixed and stained.

Results. Cells were irradiated at various times between mid-metaphase and the period in telophase during which a new resting nucleus is reconstructed. In some experiments the irradiated volume included the chromosomes; in others the radiation was confined to part of the cytoplasm. When cells were irradiated during anaphase and early telophase, in certain experiments one of the separating groups of daughter chromosomes was left unirradiated.

If the region containing the chromosomes was irradiated at metaphase, quite small doses (from 0.1 alpha particle per square micron upward) caused chromosomes to adhere, so that sticky bridges were formed between the two separating groups at anaphase. These bridges were not produced by irradiation of the cytoplasm alone, even if much larger doses were used; for instance 6 particles/μ^2 at a point near the pole of the metaphase spindle, 5μ from the nearest chromosomes. Similar results were reported by Zirkle and Bloom[9] when they irradiated amphibian cells with microbeams of protons.

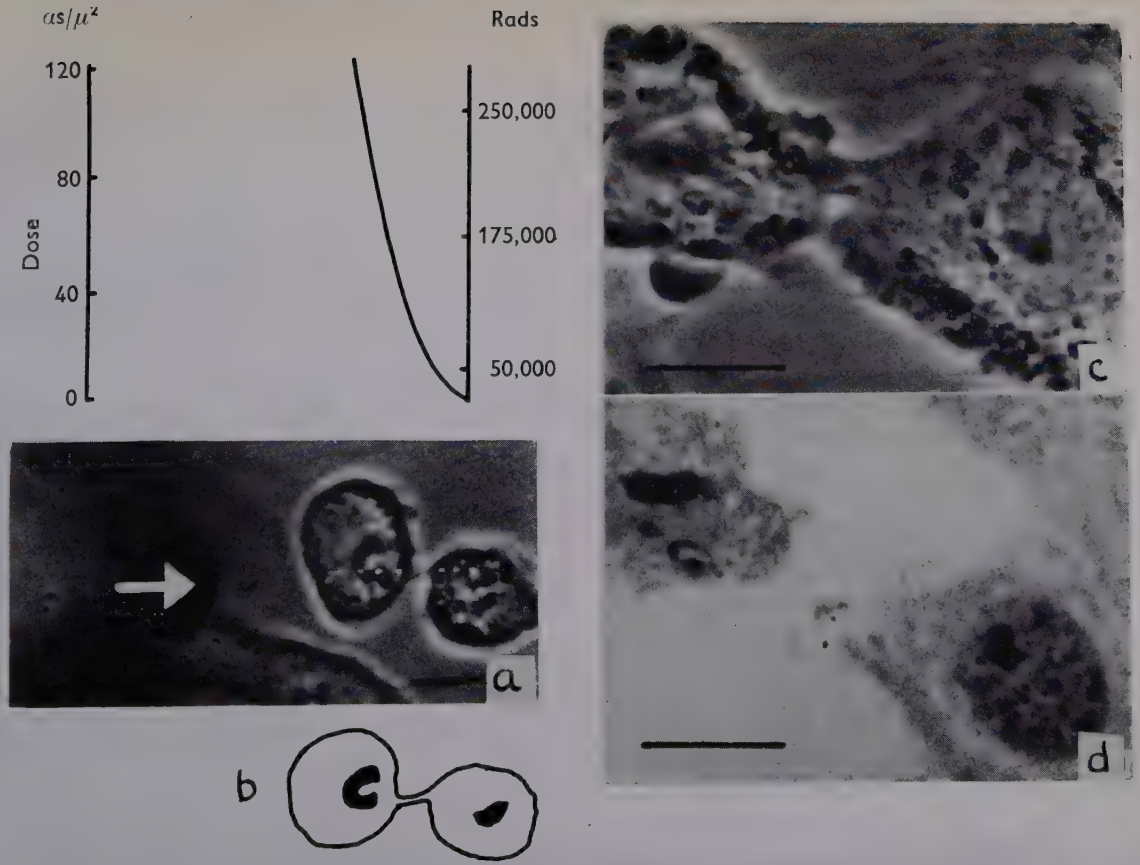


FIGURE 1. (*a*) Irradiation of one anaphase group of chromosomes in a chick fibroblast, 4 min. after anaphase began. The left hand (l.h.) group of chromosomes receives a large dose of alpha radiation from the microneedle (*marked with arrow*); the group on the right (r.h.) is not touched. The graph gives the distribution of dose along the dotted line. The black line represents 10 μ. (*b*) Diagram showing the position of the chromosomes. (*c*) Twenty-one min. after anaphase began. The r.h. daughter chromosomes have reconstructed a normal nucleus; the l.h. group has failed to reconstruct, and its chromosomes are clumped together. (*d*) Fixed and stained, 34 min. after anaphase began. Repro-

As a preliminary to an investigation of the effects of alpha irradiation upon nuclear reconstruction at telophase, the progress of normal telophase in chick fibroblasts was studied. During metaphase and early anaphase the chromosomes could be seen as discrete objects that moved independently of each other. Later in anaphase they lost their individuality and became clumped into an apparently structureless mass. They remained in this state until suddenly, 8 min. after anaphase had begun, the mass expanded and could be seen to be surrounded by a nuclear membrane, while inside the nucleus the chromosomes again became visible. After this the nucleus gradually enlarged, while the chromosomes faded away into interphase chromonemata. Chick fibroblasts at these stages must be studied in stained preparations, since granules in the cytoplasm obscure the transition from late anaphase chromosomes to a new resting nucleus; the process has been studied in newt fibroblasts, which are much more favorable material, by Boss[10] who has published beautiful photographs of living cells that show clearly how the chromosomes clump after anaphase and become discrete again as the new interphase nucleus forms. Boss measured the duration of several stages in the process, but did not report on the interval between the beginning of anaphase and the first appearance of the nuclear membrane, the constancy of which at 8 min. was such a feature of the present study.

When the chromosomes were irradiated with moderate doses (from 11 particles/μ^2 upward) during metaphase or early anaphase, nuclear reconstruction was inhibited; the chromosomes in cells fixed more than 20 min. after the beginning of anaphase still appeared sticky and clumped together, with no sign of the reappearance of a nuclear membrane. As with the production of sticky bridges at anaphase, this inhibition of telophase appeared to be mediated by a direct action of the radiation upon the chromosomes; it could not be produced by irradiation of part of the cytoplasm at metaphase with a dose as high as 34 particles/μ^2 at a point distant 5 μ from the chromosomes, near one pole of the spindle. Moreover when one group of chromosomes was irradiated during anaphase and the other left unirradiated, reconstruction appeared to take place normally in the unirradiated daughter group, even when the dose delivered to the irradiated group was as high as 85 particles/μ^2 (FIGURE 1).

To find out whether there was any change in sensitivity during the period between anaphase and the reappearance of the nuclear membrane, cells were irradiated at various stages between these limits. It seemed logical to expect that similar doses would be needed to inhibit reconstruction during the whole of the apparently quiescent period between the end of anaphase and the moment of reconstruction, while the chromosomes were clumped together. This was not the case however; the sensitivity to inhibition of reconstruction dropped between 2 and 3 min. before the nuclear membrane reappeared. During this period, doses of 16 to 117 particles/μ^2 at the chromosomes failed to inhibit reconstruction, whereas 11 particles/μ^2 had been sufficient earlier. Evidently the radiosensitive stage in telophase had been passed 2 or 3 min. before the nuclear membrane reformed, as even large doses delivered after this stage did not inhibit reconstruction. The data on the sensitivity of nuclear reconstruction to irradiation at various stages are summarized in FIGURE 2.

Not enough is known of the biochemical events during telophase to enable one to hazard a guess at the nature of the change that causes the radiosensitivity to drop. Neither the photographs of living newt fibroblasts by Boss,[10] nor my pictures of stained chick fibroblasts[11] yield any evidence as to the nature of the processes that occur during the period when the chromosomes are clumped together.

Method for Studying Cell Survival after Localized Irradiation

Studies of the effects of ionizing radiations delivered to parts of cells in tissue cultures have been concerned with morphological changes following irradiation with fine microbeams of protons[12] and alpha particles[13,14] and changes in the ultraviolet absorption of nuclei several hours after irradiation of their

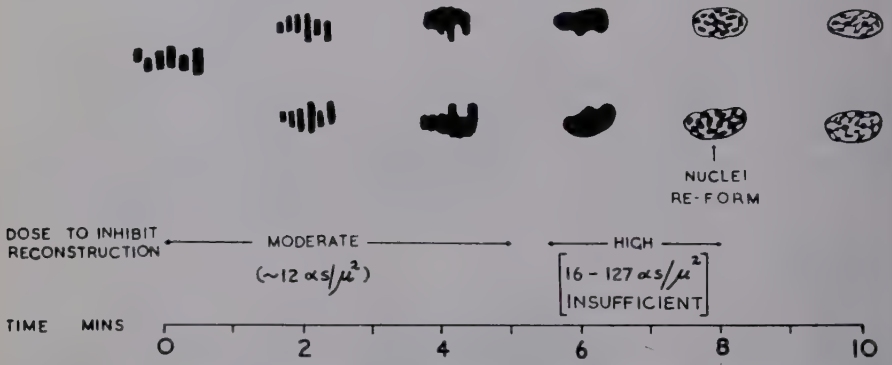


FIGURE 2. Diagram of anaphase and telophase in chick fibroblasts showing how the sensitivity to inhibition of nuclear reconstruction drops during the part of telophase when the chromosomes are clumped, approximately 2 min. before the nuclear membrane reappears. Time is measured from the beginning of anaphase.

nucleoli with a fine microbeam of X rays.[15] In all these experiments a long-term study of the fate of irradiated cells was difficult. Continuous observation was necessary because the cells were in crowded cultures and tended to migrate; consequently only limited numbers of cells could be studied.

Technique. The technique developed in this section allows a long-term study to be made of isolated single cells and their progeny and, when used in conjunction with the polonium-tipped microneedles, also permits a large proportion of the cytoplasm to be irradiated. It depends upon the fact that single isolated HeLa cells of clone S3 (Puck),[16] when seeded in suitable media, divide and grow into colonies without showing any tendency to migrate. If about 30 single cells are implanted on a cover slip and mounted on a modified chambre à huile that provides sufficient medium for a number of days' growth,[17] the positions of the cells can be mapped soon after implantation, and subsequently the same cells or their progeny can be located in the same positions; in this manner it is possible, with single daily observations, to follow individual cells as they multiply and grow into large colonies over periods of about a week. Part of a single cell on a cover slip can be irradiated with a polonium-

tipped microneedle, and its subsequent behavior can be followed for a number of days.

The microneedles can only be used to irradiate in one direction, and consequently will only irradiate the cytoplasm at one end of a cell at a time. However, since the individual cells are some distance apart on the cover slip and relatively easy to find, most of the cytoplasm of a suitably oriented spindle-shaped cell can be irradiated by exposing one end and then turning the culture through 180° on the microscope stage and irradiating the other end (FIGURE 3).

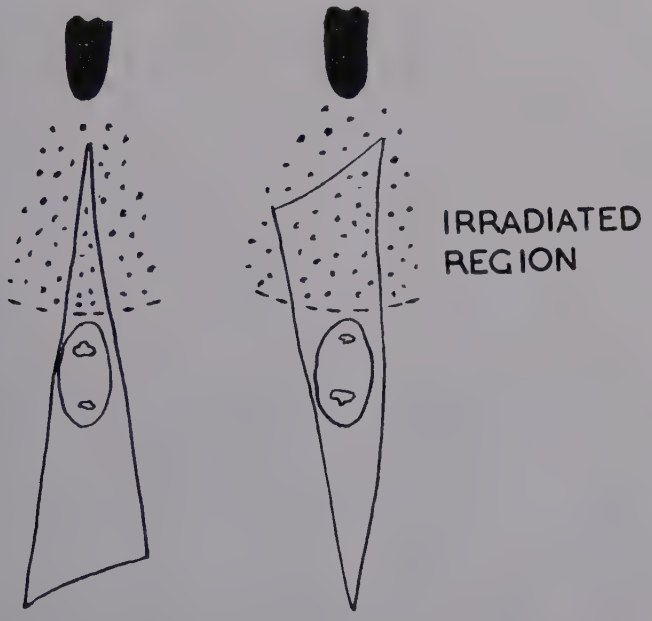


FIGURE 3. Alpha irradiation of most of the cytoplasm of a fibroblastic cell, one end at a time, from a polonium-tipped microneedle. Between exposures, the preparation is reversed on the stage of the microscope.

The method as described thus far is not quite adequate for the irradiation of parts of single isolated cells; when the cells are implanted they frequently remain rounded for some time before they flatten and spread out on their cover slips. Until a cell has spread, irradiation of its cytoplasm without the nucleus is impossible, as rounded cells do not present a sufficient area of cytoplasm. This problem has been solved by allowing cells to grow for 2 or 3 days; by that time nearly all of them have formed colonies of 4 to 8 flattened cells. All of the colonies are mapped in the manner described earlier,[17] and those that do not appear to be suitable are removed from the cover slip with a plain tungsten microneedle. Colonies are rejected if they contain too few cells or any giant or multinuclear variants, if the cells vary widely in size, if they have failed to spread, of if any cell appears abnormal.

In each of the remaining colonies, every cell except one is removed with a

microneedle. The selected cell must be well spread, fibroblastic in shape, and oriented in a direction that allows the cytoplasm at both ends to be irradiated with a polonium-tipped microneedle (FIGURE 3). Thus isolated cells that are spread out and correctly oriented for irradiation are obtained.

The cells in colonies from which single cells are isolated often adhere, so that removal of a number of cells inflicts mechanical trauma upon the one left behind. They survive this surprisingly well, and while occasional cells are rendered incapable of division, the majority grow very regularly. FIGURE 4

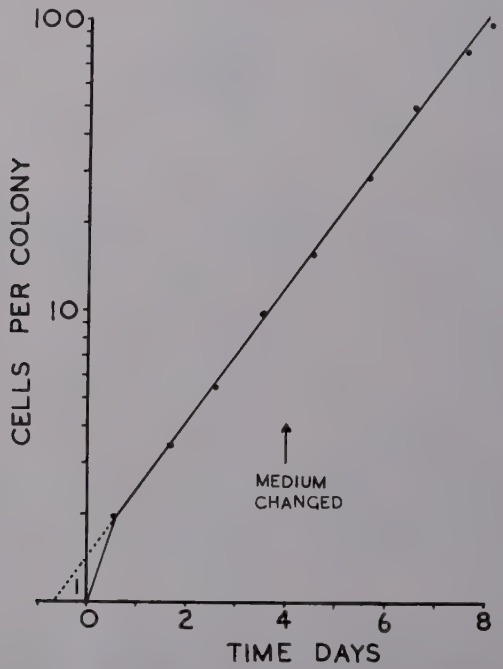


FIGURE 4. Graph of the (geometric) mean number of cells against time in 14 S3 HeLa cells as they grew into colonies. The single cells were isolated from small colonies as described in the text.

shows how the logarithm of the geometric mean of the number of cells in 14 colonies increased over a period of 8 days. After the first day the graph is a straight line that cuts off a negative intercept of 16 hours on the time axis. A similar "negative lag" was obtained in several experiments; the reason for it is probably that large, well-spread-out cells were selected to be left, and these are evidently approaching the end of interphase.

The behavior of isolated cells. When S3 HeLa cells were grown on modified chambres à huile in this manner, if conditions were favorable, about 80 per cent of the single cells divided and grew into colonies of over 50 during a period of 8 days (FIGURES 4 and 5). The majority grew regularly like cells 1 and 2 in FIGURE 6, but most of the colonies contained a few degenerating cells by the time they had grown to a moderate size. Some colonies reached a stationary

phase or even decreased in number after a few days; they could often, but not always, be induced to resume proliferation by changing the culture medium (cf. cell 3 in FIGURE 6). Others grew only slowly (cell 4) or not at all. Presumably the colonies that stopped growing but were stimulated by a change of medium had more exacting metabolic requirements than the majority of the cells in the culture.

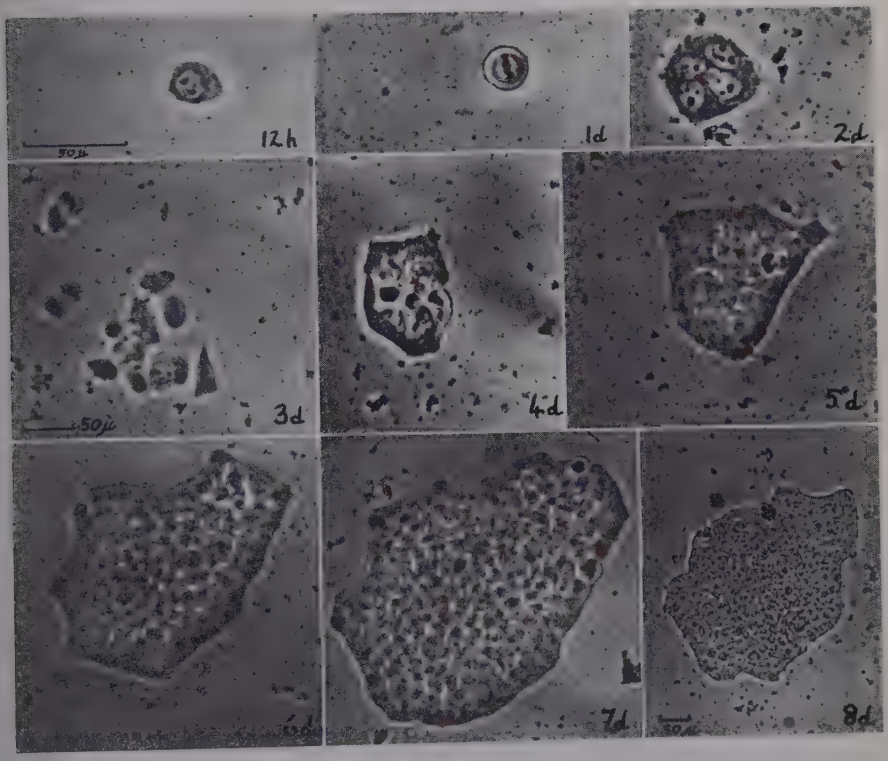


FIGURE 5. Photographs taken at low powers of a single S3 HeLa cell and its progeny after periods of growth of 12 hours to 8 days. Note metaphase at 1 day; synchronous division in progress at 3 days. Key: *d*, day; *h*, hour.

The behavior of the cells that did not grow into large colonies was of interest. Some appeared quite normal but did not divide, or divided only a few times. Multinucleate cells of several kinds were seen; some had 2 or more nuclei that appeared normal and probably resulted from mitoses that were normal except for the absence of cytoplasmic cleavage. Others had small nuclei, or nuclei that contained very abnormal inclusions. One class of multinucleate cell was large and the cytoplasm was flattened and circular; the number of nuclei present increased rapidly until after 3 to 4 days there might be 12 to 16, so that little cytoplasm was visible and the cell appeared full of nuclei. After this stage had been reached, such cells usually vanished without trace. Another characteristic cell type comprised large, very flattened cells, in which

both cytoplasm and nucleus showed remarkably little contrast when viewed by phase. These appeared lethargic and never grew into colonies although they occasionally divided once or twice. They frequently degenerated during the 8 days for which cultures were observed.

The technique allowed observation of the manner in which cells degenerated. In these untreated cells, the time between the onset of degeneration and the stage by which the cell had been reduced to a collection of fatty-looking glob-

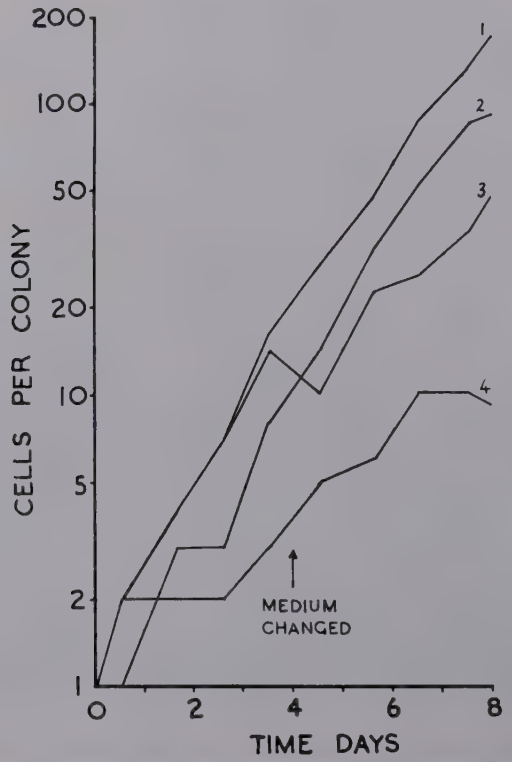


FIGURE 6. Growth curves for four of the individual S3 HeLa cells from which FIGURE 4 was derived.

ules was usually 24 to 48 hours. The method could be used with advantage to study the manner in which cells degenerate after irradiation.

Discussion

The short-term experiments described above under *Localized Irradiation of Single Cells in Culture* indicate that the damage to the chromosomes and chromosomal mechanisms, produced and observed during mitosis, was due to a direct effect of the radiation acting on or very near the chromosomes, and was not mediated via release of depolymerizing enzymes from the cytoplasm or damage to its function. While the changes described were not concerned with the long-term lethal effects of the radiation, they cannot be explained in terms

of Alexander's recent suggestion[18] that the phospholipids of intracellular membranes are an important site of the action of ionizing radiations.

Boss's observations on telophase[10] suggest that telophase chromosomes must shed material before the nuclear membrane can be reconstructed. The present experiments show that alpha irradiation during metaphase or anaphase interferes with this shedding process and promotes chromosomal adhesion. The sticky bridges seen at anaphase are a result of abnormal adhesion; the continued clumping of the chromosomes at telophase when irradiation has inhibited reconstruction may be mediated via a similar process, or by an inhibition of the mechanism by which the interchromosomal material that

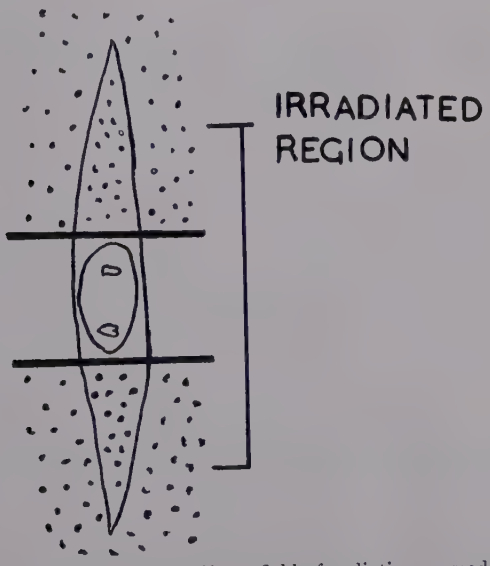


FIGURE 7. The manner in which a uniform field of radiation crossed by a shielded strip could be used to irradiate the cytoplasm of a fibroblastic cell.

normally causes adhesion at telophase is removed. The finding that the last 2 min. of telophase were not sensitive to alpha irradiation suggests that some process preceding the dissolution of the clumps of chromosomes and the formation of the nuclear membrane is inhibited by the radiation.

As yet there are no reports that demonstrate unequivocally that it is, in animal cells, the effect of ionizing radiations on the nucleus rather than the cytoplasm that causes such cells to lose their reproductive integrity; the nearest approach is in the work of Drew and Painter[19] on the survival of S3 HeLa cells following nuclear irradiation by incorporated tritiated thymidine; this is not sufficient evidence because it has not yet been possible to calculate the dose of radiation delivered by the intranuclear tritium. An adequate demonstration would require (1) a tissue culture method that, like the one employed by Drew and Painter, made possible the irradiation of parts of isolated single cells growing under conditions that allowed them to divide and form colonies, and (2) a technique for irradiation that allowed a measured dose of radiation to be

delivered to a large proportion of the cytoplasm of a cell, without the nucleus. If, as in the Habrobracon eggs referred to earlier, only a proportion of the cytoplasm is irradiated, the remainder may be able to take over the function of the damaged region.

The problem of irradiating most of the cytoplasm without the nucleus could be attacked by designing apparatus that produced a uniform field of radiation crossed by a shielded strip 12 to 15 μ wide that could be used to keep the radiation from the nucleus of a long fibroblastic cell (FIGURE 7). Such a "microshadow" apparatus might yield results as interesting as those from irradiation with fine microbeams.

Summary

Some experiments on the irradiation of parts of dividing chick fibroblasts with alpha particles from polonium-tipped microneedles are described. Chromosomal abnormalities were seen if the chromosomes were irradiated, but not if the radiation was confined to part of the cytoplasm. Other experiments on the irradiation of parts of single cells are reviewed, with emphasis on work with cultured animal cells, and it is concluded that evidence for the belief that the effects of ionizing radiations upon the nuclei, rather than the cytoplasm, are responsible for depriving mammalian cells of reproductive integrity is not yet adequate. A method is described that allows a large fraction of the cytoplasm of single S3 HeLa cells to be irradiated without the nucleus. The cells and their progeny can subsequently be followed for more than one week.

Acknowledgments

I acknowledge my debt to the encouragement I received from Edith Paterson at the Christie Hospital, Manchester, England, and I am grateful to H. B. Fell for her careful revision of the manuscript.

References

1. VINTEMBERGER, P. 1928. Sur les effets d'applications de rayons X localisés soit au protoplasme, soit à la region nucléaire de la cellule. Compt. rend. soc. biol. **99:** 1968.
2. ASTAUROV, B. L. & V. P. OSTRIAKOVA-VARSHAVER. 1957. Complete heterospermic androgenesis in silkworms as a means for experimental analysis of the nucleus-cytoplasm problem. J. Embryol. Exptl. Morphol. **5:** 449.
3. ROGERS, R. W. & R. C. VON BORSTEL. 1957. Alpha-particle bombardment of the Habrobracon egg. I. Sensitivity of the nucleus. Radiation Research. **7:** 484.
4. VON BORSTEL, R. C. & R. W. ROGERS. 1958. Alpha-particle bombardment of the Habrobracon egg. II. Response of the cytoplasm. Radiation Research. **8:** 248.
5. ANIANSSON, G. 1954. New method for measuring the α-particle range and straggling in liquids. Phys. Rev. **98:** 300.
6. MUNRO, T. R. 1958. Alpha irradiation of parts of single cells in tissue cultures. I. Techniques. Exptl. Cell Research. **15:** 529.
7. MUNRO, T. R. 1958. Alpha irradiation of parts of single cells in tissue cultures. II. Dosimetry. Exptl. Cell Research. **15:** 537.
8. COMMANDON, J. & P. DE FONBRUNE. 1938. La chambre à huile. Ses avantages pour l'étude des microorganismes vivants, la culture des tissus et la micromanipulation. Ann. Inst. Pasteur. **60:** 113.
9. ZIRKLE, R. E. & W. BLOOM. 1953. Irradiation of parts of individual cells. Science. **117:** 487.
10. BOSS, J. M. N. 1959. The contribution of the chromosomes to the telophase nucleus in cultures of fibroblasts of the adult crested newt Triturus Cristatus Carnifex. Exptl. Cell Research. **18:** 197.

11. Munro, T. R. 1959. Alpha irradiation of parts of single cells in tissue cultures. III. Irradiation of chick fibroblasts during metaphase and anaphase. Exptl. Cell Research. **18:** 76.
12. Bloom, W. & R. E. Zirkle. 1955. Irradiation of parts of individual cells. III. Effects of chromosomal and extrachromosomal irradiation on chromosome movements. Ann. N.Y. Acad. Sci. **59**(4): 503.
13. Davis, M. & C. L. Smith. 1957. The irradiation of individual parts of single cells in tissue culture with a microbeam of α-particles. I. Apparatus. Exptl. Cell Research. **12:** 15.
14. Smith, C. L. 1959. The irradiation of the chromosomes of single cells in metaphase with a microbeam of α-particles 5 μ in diameter. Proc. Roy. Soc. B. **150:** 372.
15. Seed, J. 1960. Inhibition of nucleic acid synthesis caused by X-irradiation of the nucleolus. Proc. Roy. Soc. B. **152:** 387.
16. Puck, T. T., P. I. Marcus & S. J. Ciecura. 1956. Clonal growth of mammalian cells *in vitro*. J. Exptl. Med. **103:** 273.
17. Munro, T. R. 1960. A technique that permits micromanipulation and long-term study of single HeLa cells and their progeny. Exptl. Cell Research. **19:** 291.
18. Alexander, P. 1961. Report of congress on primary and initial effects of ionizing radiations. Nature. **189:** 110.
19. Drew, R. M. & R. B. Painter. 1959. Action of tritiated thymidine on the clonal growth of mammalian cells. Radiation Research. **11:** 535.

THE GRASSHOPPER NEUROBLAST CULTURE TECHNIQUE AND ITS VALUE IN RADIOBIOLOGICAL STUDIES

J. Gordon Carlson

Department of Zoology and Entomology, University of Tennessee, Knoxville, Tenn.

It is a well-known fact that much greater success has attended the development of culture methods for vertebrate than for invertebrate tissues. While the culturing of the former has become a routine technique, invertebrate tissues have thus far defied the best efforts of many researchers to grow them *in vitro* with anywhere near the same favorable results (Day and Grace, 1959). It might be questioned, and justifiably so, whether the invertebrate technique I am about to describe should be honored with the word "culture." In our best preparations a neuroblast may undergo successive divisions at a relatively uniform rate through a total of 6 mitotic cycles over a period of 5 to 6 days at 26° C. Even after mitosis ceases, however, most of the embryonic cells live and undergo differentiation. This is strikingly illustrated by the increasing pigmentation along the sides of the embryo, which continue to become darker for many days after mitosis has ended. Our interest, however, has centered primarily in neuroblast mitosis and the effects of different kinds of radiations on mitosis and on the structure and behavior of various parts of the dividing cell. The neuroblast technique, although inferior in some respects to vertebrate techniques, is admirably suited to our purposes and has given us the answers to many questions.

On the assumption that our technique is little known to most persons working in the tissue culture field I shall devote most of this paper to the technique and its value in radiobiological studies, including only a summary of the chief contributions it has made to our knowledge of radiation effects.

Material and Technique

The species of grasshopper we use, *Chortophaga viridifasciata* (De Geer), is the one North American grasshopper that does not have a diapause; therefore adults can be collected in the field during the spring and fall in the north and, in addition, during midsummer in the south. The range of this species includes eastern North America from New England, Ontario, and Saskatchewan on the north to Georgia, Oklahoma, and Texas on the south. Other species are suitable in so far as the technique and the cytological favorability of the material are concerned, but adults and, therefore, eggs of these can be obtained in the field only in the late summer and fall.

Adults are kept in cages containing small dishes with firmly-packed moist sand to lay their eggs in. The eggs, which are laid in pods containing 15 to 30 eggs each, are removed once or twice a week and stored at 14° C. in Petri dishes containing moist sand. They can be kept for several months at this temperature without developing beyond the stage at which we use them in hanging drops. As needed they are exposed to room temperature or to a maximum of 38° C. to bring development to the optimum stage for study, that is, the equivalent of 14 days' development at 26° C. or about one week at 38° C.

(FIGURE 1). Because all the eggs of one pod are fertilized and laid within a few minutes of each other, the stage of development of all may be determined by checking one egg.

An embryo of the desired age is placed in ultraviolet-sterilized culture medium (Carlson *et al.*, 1947), the chorion and yolk are removed, the amniotic membrane is ripped open, and the thoracic appendages, the head anterior to the maxillae, and the abdomen posterior to the third or fourth abdominal segment are cut off. The remaining portion is placed ventral surface against a cover glass in a small film of culture medium containing some grasshopper egg yolk. This is inverted over a depression slide and sealed with mineral oil.

Until recently the culture solution we used consisted of organic salts and glucose (Carlson, 1946; Carlson *et al.*, 1947). This is made up as needed from three stock solutions: (1) 7 per cent NaCl, 0.2 per cent KCl, 0.2 per cent $CaCl_2$, 0.1 per cent $MgCl_2$, 0.2 per cent NaH_2PO_4; (2) 0.5 per cent $NaHCO_3$, if to be autoclaved, or 0.12 per cent $NaHCO_3$, if to be sterilized with ultraviolet radiation; (3) 8 per cent dextrose. One part each of these three solutions is added to approximately 7 parts of Pyrex-distilled H_2O before use. More

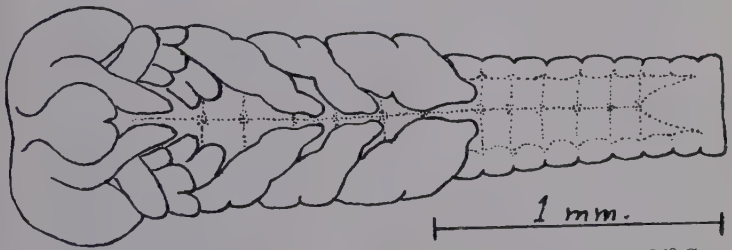


FIGURE 1. *Chortophaga* embryo at an age equivalent to 14 days at 26° C.

recently we have been using a solution formulated by Shaw (1956*a*) in which glutamate and glycine are substituted for the NaCl and KCl to bring the sodium-to-potassium ratio and the concentrations of chlorides and organic anions in closer agreement with those existing in the insect. It is prepared from these stock solutions: (1) 0.5 *M* potassium glutamate plus 0.5 *M* glycine (7.35 g glutamic acid, and 3.75 g glycine adjusted to *p*H 7.3 by the addition of concentrated KOH free of bicarbonate, and diluted with Pyrex-distilled water to make 100 ml. of solution); (2) 0.5 *M* sodium glutamate plus 0.5 *M* glycine (prepared in the same manner as solution 1 except that the *p*H is adjusted with NaOH); (3) 0.2 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, Pyrex-distilled H_2O to make 100 ml.; (4) 0.8 g NaH_2PO_4, 0.5 g $NaHCO_3$, Pyrex-distilled H_2O to make 100 ml.; (5) 7.0 g anhydrous glucose (or 7.7 g glucose$\cdot H_2O$); Pyrex-distilled H_2O to make 100 ml. The culture medium is prepared by mixing 0.75 ml. of 1, 1.25 ml. of 2, 1.0 ml. each of 3, 4, and 5, and approximately 5.0 ml. of Pyrex-distilled H_2O. With either medium a small amount of grasshopper egg yolk is placed in the hanging drop (Gaulden and Kokomoor, 1955). The amounts of water with which the stock solutions are diluted is given in approximate amounts because the culture solution must be individually adjusted to be isotonic with the embryonic cells of each different egg pod.

The technique described can be adapted to the study of different kinds of radiations. In X-ray and gamma-ray experiments the cells may be irradiated within the intact egg chorion and made into a hanging-drop preparation a selected interval of time later, or they may be irradiated in a hanging drop. For alpha particles it is necessary to substitute thin mica for the cover glass (Rogers, 1955). For beta radiation the embryo is oriented with the ventral side downward against a rubber hydrochloride membrane forming the bottom of a small dish containing culture medium and treated by inserting a P^{32} Bakelite plaque beneath it (Gaulden *et al.*, 1952). After irradiation the embryo is mounted in a hanging-drop for observation. For ultraviolet radiation a quartz cover is substituted for the glass cover, and the neuroblasts on one side of the mid-ventral line of the embryo are shielded with film or black paper to serve as controls (Carlson and Hollaender, 1944).

Advantages of the Technique

Hanging-drop preparations of grasshopper embryos have a number of advantages as material for the study of radiation effects on cell morphology, mitosis, and related cellular phenomena.

(1) With planning, one can have living material available throughout the year. By collecting the adults or older nymphs in spring and again in early fall, keeping them in cages, collecting the eggs once a week from the moist sand in which they are laid, and storing them at 14° C., development can be kept almost at a standstill for months and large numbers of eggs can be accumulated. When eggs are wanted, they can be placed at higher temperatures (up to 38° C.) to bring them quickly to the desired stage of development.

(2) A major advantage of the grasshopper neuroblast and one of the reasons we were interested from the start in developing a culture technique was the large size of this cell and its chromosomes. Neuroblasts measure approximately 25 μ in diameter at metaphase. The metaphase chromosome diameter is 1.5 μ, and the longer chromosomes measure 12 to 15 μ in length. Most features of the cell that are visible in fixed and stained preparations can also be seen in the unstained, living cell; to name a few: the nucleoli, including their appearance at telophase, their increase in size and change in shape, and their disappearance at late prophase; the chromosomal constrictions with which the nucleolar organizers are associated; disappearance of the nuclear membrane at the end of prophase and its reappearance in telophase; the identification, chiefly from nuclear contents, of at least 14 distinct stages in the mitotic cycle; the spindle and its changes from its formation and development in prometaphase to its disappearance in telophase; and mitochondria and their Brownian movement. The centrosomes cannot be distinguished in the living cell.

(3) At the stage of embryonic development when the neuroblasts are largest and most active mitotically, they are very close to the surface of the embryo (FIGURE 2). This stage, which is the equivalent of 14 days development at 26° C., represents about one third of the time from egg laying to hatching. The neuroblasts of the thoracic region are covered only by a thin layer of hypodermal cells. These are so thin in relation to the neuroblasts that in hanging-drop preparations many of the neuroblasts appear to be in contact

with the cover glass. Only when the nucleus of a hypodermal cell lies directly ventral to a neuroblast does a detectable distance separate it from the cover glass, and the use of such neuroblasts can be avoided, if necessary. There are two main advantages in using cells close to the cover glass: (*a*) selected cells can be examined repeatedly with the oil immersion objective of an ordinary light microscope over hours or days and the internal structural details remain sharp and clear, and (*b*) if a cover of quartz, mica, or rubber hydrochloride instead of glass is used, poorly penetrating radiations such as ultraviolet, alpha particles, or beta particles may be used with relatively little loss of energy between the source and the neuroblast.

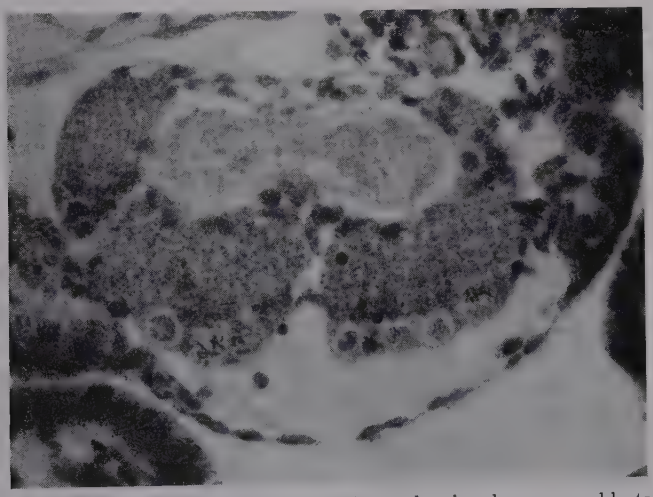


FIGURE 2. Cross section of grasshopper embryo showing large neuroblasts above the thin layer of hypodermal cells (*at bottom*). Dorsal to the neuroblasts are the ganglion cells, the oldest of which have developed nerve fibers (*above center*). Extending upward from each neuroblast is the chain of ganglion cells derived from it. The space separating the hypodermal cells and neuroblasts is obliterated by the pressure of the cover glass in the hanging-drop preparation.

(4) Each neuroblast maintains a relatively constant location with respect to other neuroblasts of the same segment. The neuroblast divides unequally, giving rise to a large ventral daughter neuroblast and a small dorsal ganglion cell. The consequence of repeated unequal divisions of these cells is, therefore, the formation dorsally of more or less parallel chains of ganglion cells with a single neuroblast at the ventral end of each chain (FIGURE 2). The neuroblasts tend to be arranged in columns running laterally from the midventral line and in rows running from anterior to posterior (FIGURE 3). Over the course of several days and several divisions a given neuroblast may shift position some with respect to neighboring neuroblasts but is unlikely to leave its row and column. Neuroblasts near the anterior, lateral, and posterior edges of each segment may be too far from the cover glass to be usable; there usually are, however, about 5 columns and 4 rows of usable neuroblasts, and these can each be designated by a number. This makes it possible to follow concurrently

the course of division of a number of selected cells in a number of different embryos over the course of several hours or several days. Our system, for example, has been to designate the first maxillary through the first abdominal segment by the numbers 1 through 6; therefore neuroblast 5R32 would be the second cell from the mid-line in the third column from the anterior edge of the right side of the third thoracic segment. The system is sufficiently dependable and accurate that M. E. Gaulden and I, when working on a joint research project, have been able to use each other's slides and data sheets in locating cells and recording observations of radiation effects.

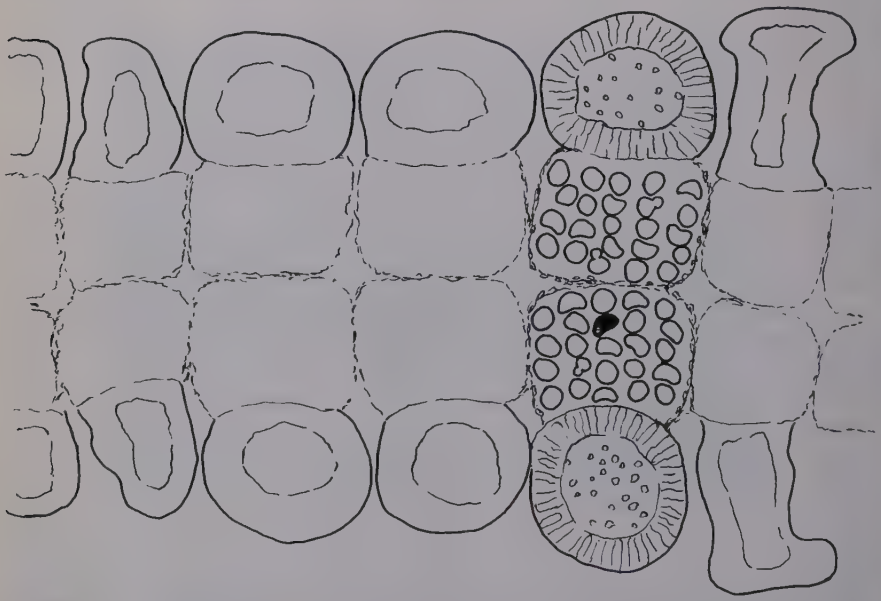


FIGURE 3. Diagram of ventral surface of a portion of grasshopper embryo from first maxillary (partly missing) at left through first abdominal segment at right, which we designate as segments 1 to 6 respectively. The cell in solid black would be designated 5R32, because it is in segment 5, on the right side, in the third column from the anterior border of the segment and is the second cell from the midventral line. Redrawn from a sketch by M. E. Gaulden.

(5) With practice one can learn to identify in fixed and stained whole mounts individual cells whose previous mitotic history is known from observation in hanging-drop preparations. This is specially valuable when one desires to emphasize some particular X-ray-induced alteration that is not sharply defined enough for careful observation and study in the living cell. A more difficult, but still possible, procedure is to identify in fixed and stained serial sections particular cells that were previously observed in the living state. This technique has been utilized in radioisotope studies by Gaulden, who has exposed cells in hanging drops to tritiated thymidine, followed these cells to the desired mitotic stages, and then fixed, sectioned, stained, and made radioautographs, in which she could identify and observe the radioactivity in the same cells.

(6) Through the 14-day stage most of the neuroblasts divide with the axes of the mitotic spindles oriented approximately dorsoventrally so that the ganglion cell is formed dorsal to the neuroblast. As the age of the embryo increases many of the ganglion cell chains become bent to one side at their ventral ends, the neuroblast tends to lie slightly to one side of the ganglion cells, and the axis of its mitotic spindle lies more or less parallel to the ventral surface of the embryo. If, therefore, one wishes to study the metaphase chromosomes carefully and desires to see these in polar view, it is best to use embryos somewhat on the young side of 14 days; on the other hand, if one desires to study radiation-induced fragments at anaphase, embryos older than 14 days with a larger proportion of side views are to be preferred.

(7) Unlike most kinds of cells that divide to form two equal-sized cells and so double their numbers with each new cell generation, the neuroblasts, because the parent cell gives rise to only one daughter cell like itself, maintain a constant number during the period when they are producing the cells of the central nervous system, at least in so far as any given segment of the organism is concerned. This is distinctly advantageous to the investigator who desires to determine the effects of radiation on mitosis by making counts of the cells in different stages at certain intervals of time after treatment. No correction need be made for increase in the number of cells in the group being counted, or, if the cells are being counted in a given area, compensation need not be made for changes in cell density. If the time required by the cell to progress through the stage being counted is known, a series of successive counts made at this interval of time gives the total number of cells passing through this stage in the total period over which the counts extend. The investigator must make certain, however, that the mitotic stage used is not itself delayed or accelerated by the irradiation. In the grasshopper neuroblast we have used chiefly the prometaphase-metaphase-anaphase combination of stages, for this mid-mitotic stage is readily identifiable in rapid surveys of the cells, and is highly resistant to ionizing radiations. The average duration of this stage at 38° C. is 22 min.; therefore we have used this counting interval in many of our studies.

Shortcomings of the Technique

Our method, like others, has certain shortcomings, and it is only fair to point these out also:

(1) The thickness of the embryo, while it does not interfere appreciably with observation with the ordinary light microscope, prevents the use of phase-contrast microscopy to full advantage. Embryos can be squashed to the thickness of a single neuroblast, but this apparently results in some cell injury, for mitosis is retarded and soon ceases. The embryonic cells can also be separated by mechanical agitation following immersion in culture fluid containing hyaluronidase and trypsin, and these can be suspended in a hanging drop (St. Amand and Tipton, 1954; Gaulden and Perry, 1958). The neuroblasts in such preparations, however, instead of being concentrated in one area where they are easily found, are scattered among the much more numerous nonneuroblasts and so are hard to find. It is even more time-consuming to

find them in a particular desired stage. In such preparations mitosis proceeds more slowly than when the cells are left in their natural relationships.

(2) Mitotic activity in the grasshopper neuroblast is quite sensitive to changes in osmotic pressure, and eggs from different pods may differ with respect to osmotic pressure. It is necessary, therefore, in the more exacting types of experiments to adjust the osmotic pressure of the culture medium individually for each egg pod. This is done by making a hanging-drop preparation of one egg of a pod, allowing a few minutes for the cells to adjust to the surrounding medium, and then examining it under the microscope to see if it is hypertonic or hypotonic. If it is slightly hypertonic the metaphase chromosomes appear unusually sharp in outline, clear vacuolelike bodies arise in the protoplasm among the outer ends of the metaphase chromosomes, the nucleoli are invisible or barely visible, and mitosis is accelerated. If the solution is strongly hypertonic the chromosomes clump at metaphase, sister chromatids adhere at anaphase, and the mitotic rate is slowed. If it is hypotonic the nucleoli are unusually clear and sharp in outline, the metaphase chromosomes are barely visible, and mitotic progress is retarded temporarily. Even very slight temperature changes of the air surrounding the slide must be guarded against, for the cover glass and hanging drop undergo temperature changes more rapidly than the thicker slide below, which can lead to osmotic changes from differential evaporation and condensation within the moist chamber. Precautions must be taken to prevent this when the slide is moved from an area at one temperature to an area at another temperature. If the slide is to be studied outside an incubator the microscope stage must be shielded from moving air currents and from any sources of irradiated heat, such as the microscope lamp and the body and breath of the observer.

Summary of Radiation Effects

The effects of ionizing and ultraviolet radiations on the grasshopper neuroblast have been described in a number of papers and reviewed recently (Carlson, 1954, 1958) and therefore will only be summarized here.

The following results have been obtained from studies of grasshopper neuroblast cultures treated with ionizing radiations:

(1) The decrease in the number of cells passing through metaphase and anaphase soon after treatment with ionizing radiations is due to temporary mitotic blockage of cells at late prophase a few minutes before breakdown of the nuclear membrane (Carlson, 1941, 1950; Carlson *et al.*, 1953). Small doses of X rays (for example, doses of 16 r and less) merely stop or retard most cells at middle and late prophase. Doses of 32 r and greater, however, cause certain cells in middle and late prophase not only to stop their forward progress but to undergo mitotic reversion or a series of morphological changes simulating reversal (Carlson, 1941, 1950; St. Amand, 1956). Such cells revert to early prophase or interphase, but eventually recover and progress through mitosis. Because the blocked and reverted cells are added to those progressing with little or no delay, an abnormally large number of cells passes through metaphase and anaphase soon after recovery (Carlson and Hollaender, 1944). The mitotic order of the cells is altered as a result of the reversion. It has been found, for

example, that at 38° C. in the second hour after irradiation only cells that were in early prophase at the time of treatment enter anaphase; in the third hour cells treated in early and late telophase, interphase, and early-through-late prophase reach anaphase; while from the fourth hour on cells treated in all stages of the mitotic cycle reach anaphase (St. Amand, 1956). A dose as small as 3 r produces significant depression of mitosis (Gaulden, unpublished), and the ionizations of a single alpha particle are probably sufficient to stop the mitotic progress of a cell in middle prophase (Rogers, 1955).

(2) The mitotic effect is independent of the dose rate after the smaller doses, but a high dose rate is more effective than a low dose rate in depressing mitosis after large doses (Carlson *et al.*, 1949).

(3) When embryos within the intact egg chorion are irradiated in different concentrations of oxygen, no effect is found on the depth to which mitosis is depressed but an influence is found on the extent of recovery (Gaulden *et al.*, 1953). High oxygen concentration delays the attainment of maximum mitotic activity after X-ray-induced mitotic retardation. On the other hand, when the oxygen tension is reduced by placing dissected embryos in hydrosulfite solution prior to and during X-raying, the depression of mitotic activity by 8 r and 32 r is greatly reduced, probably because of the reduction of free oxygen concentration within the cell (Shaw, 1956*b*).

(4) When the medium formulated by Carlson (1946) is used in the hanging drop, recovery from radiation-induced inhibition of mitosis does not occur unless a small amount of grasshopper egg yolk has been added to the hanging drop, approximately one fourth of the yolk of one egg being necessary for maximum recovery (Gaulden and Kokomoor, 1955).

(5) The extent of anaphase adhesion of sister chromatids that appears within an hour of X-raying is positively correlated with the dose of radiation and with the length of the time interval between treatment and detection, that is, the larger the dose and the earlier the stage at which irradiation occurs the more extensive is the adhesion (Carlson and Harrington, 1955).

(6) Chromosome breakage to produce chromosome, isochromatid, or chromatid fragments may be induced at any stage of the mitotic cycle, although those induced in very late prophase, prometaphase, and metaphase are often not detected until the second anaphase after treatment. The induction of chromatid fragments at all stages indicates that the chromosome is at least two-stranded throughout the mitotic cycle. Studies of the frequencies of chromosome aberrations induced by X-ray treatment at different stages of mitosis show two maxima, namely, middle telophase and middle prophase, and two minima, namely, interphase, and very late prophase (St. Amand, 1956).

(7) Most cells that contain X-ray-induced fragments show a greater mitotic delay than those that lack fragments (St. Amand, 1956).

The following effects have been found to result from treatment with ultraviolet radiation:

(1) Mitosis is depressed temporarily by wave lengths 2250 and 2537 Å, primarily through retardation of the progress of cells in interphase and prophase at the time of treatment (Carlson and Hollaender, 1944, 1948). The former wave lengths causes reversion of cells in middle and late prophase.

(2) Like X rays, ultraviolet radiation of wave length 2537 Å shows an intensity effect after large doses, high intensity depressing mitosis to a greater extent than the same dose given at low intensity (Carlson and Hollaender, 1945).

(3) Neuroblasts irradiated with 2250 Å ultraviolet at very late prophase or prometaphase exhibit reduced spindle size at anaphase, the nuclear karyolymph that would normally have gone into the formation of the spindle collecting at one side, usually near a pole of the spindle, as a large hyaline globule. Treatment at metaphase or early anaphase also has a destructive action on the spindle, which is reduced in size, but no hyaline globule appears. The abnormally small spindle occupies the middle of the cell and does not shift toward the ganglion-cell side of the neuroblast during anaphase, with the result that the neuroblast frequently divides equally to form two daughter neuroblasts instead of unequally to give a large daughter neuroblast and a small daughter ganglion cell (Carlson and Hollaender, 1948). The same effect on the spindle and the position of the cleavage furrow is produced by treatment with colchicine (Gaulden and Carlson, 1951), by calcium removal, with hyaluronidase present or absent (Kawamura and Carlson, unpublished), by micromanipulation, when the spindle is held in the middle of the neuroblast with a microneedle (Carlson, 1952), and by mechanical separation of cells, when the cells normally in contact with the neuroblast on the side opposite the ganglion cells are separated from it (Kawamura and Carlson, unpublished).

(4) A number of ultraviolet wave lengths, namely, 2250, 2399, 2537, 2650, 2804, 3022, and 3130 Å, are effective in inducing nucleolar spheration. The effectiveness of wave lengths 2650–3130 Å is reduced when the neuroblasts are exposed to visible light during and following treatment (Carlson and McMaster, 1951).

(5) Microbeam irradiation of the nucleolus of the neuroblast with mixed wave lengths (Gaulden and Perry, 1958) and, with 2537 or 2804 Å, ultraviolet (Carlson *et al.*, 1961) induces mitotic delay in any stage from middle telophase through middle prophase, but small doses of 2804 Å radiation produce mitotic acceleration (Carlson *et al.*, 1961).

References

Carlson J. G. 1941. Effects of X-radiation on grasshopper chromosomes. Cold Spring Harbor Symp. Quant. Biol. **9:** 104–111.

Carlson, J. G. 1946. Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis. Biol. Bull. **90:** 109–121.

Carlson, J. G. 1950. Effects of radiation on mitosis. Jour. Cell. Comp. Physiol. **35** (Suppl. 1): 89–102.

Carlson, J. G. 1952. Microdissection studies of the dividing neuroblast of the grasshopper, *Chortophaga viridifasciata* (De Geer). Chromosoma. **5:** 199–220.

Carlson, J. G. 1954. Immediate effects on division, morphology, and viability. *In* Radiation Biology. Ed., A. Hollaender. McGraw-Hill. New York, N.Y. **1**(11):

Carlson, J. G. 1958. The immediate cytological effects of ionizing radiations. Bios. **29:** 106–117.

Carlson, J. G., M. E. Gaulden & J. Jagger. 1961. Mitotic effects of monochromatic ultraviolet irradiation of the nucleolus. Proc. 3rd Internat. Cong. Photobiol. In press.

Carlson, J. G. & N. G. Harrington. 1955. X-ray-induced "stickiness" of the chromosomes of the *Chortophaga* neuroblast in relation to dose and mitotic stage at treatment. Rad. Res. **2:** 84–90.

Carlson, J. G., N. G. Harrington & M. E. Gaulden. 1953. Mitotic effects of prolonged irradiation with low-intensity gamma rays on the *Chortophaga* neuroblast. Biol. Bull. **104:** 313–322.

Carlson, J. G. & A. Hollaender. 1944. Immediate effects of low doses of ultraviolet radiation of wavelength 2537 A on mitosis in the grasshopper neuroblast. Jour. Cell. Comp. Physiol. **23:** 157–169.

Carlson, J. G. & A. Hollaender. 1945. Intensity effects of ultraviolet radiation of wavelength 2537 Å on mitosis in the grasshopper neuroblast. Jour. Cell. Comp. Physiol. **26:** 165–173.

Carlson, J. G. & A. Hollaender. 1948. Mitotic effects of ultraviolet radiation of the 2250 Å region with special reference to the spindle and cleavage. Jour. Cell. Comp. Physiol. **31:** 149–173.

Carlson, J. G., A. Hollaender, & M. E. Gaulden. 1947. Ultraviolet radiation as a means of sterilizing tissue culture materials. Science. **105:** 187–188.

Carlson, J. G. & R. D. McMaster. 1951. Nucleolar changes induced in the grasshopper neuroblast by different wavelengths of ultraviolet radiation and their capacity for photorecovery. Exp. Cell. Res. **2:** 434–444.

Carlson, J. G., M. L. Snyder & A. Hollaender. 1949. Relation of gamma-ray dosage rate to mitotic effect in the grasshopper neuroblast. Jour. Cell. Comp. Physiol. **33:** 365–372.

Day, M. F. & T. D. C. Grace. 1959. Culture of insect tissues. Ann. Rev. Entom. **4:** 17–38.

Gaulden, M. E. & J. G. Carlson. 1951. Cytological effects of colchicine on the grasshopper neuroblast *in vitro* with special reference to the origin of the spindle. Exp. Cell. Res. **2:** 416–433.

Gaulden, M. E. & K. L. Kokomoor. 1955. Influence of yolk on mitotic rate in untreated and X-rayed grasshopper neuroblasts *in vitro*. Proc. Soc. Exp. Biol. & Med. **90:** 309–314.

Gaulden, M. E., M. Nix, & J. Moshman. 1953. Effects of oxygen concentration on X-ray-induced mitotic inhibition in living *Chortophaga* neuroblasts. J. Cell. Comp. Physiol. **41:** 451–470.

Gaulden, M. E. & R. P. Perry. 1958. Influence of the nucleolus on mitosis as revealed by ultraviolet microbeam irradiation. P.N.A.S. **44:** 553–559.

Gaulden, M. E., C. W. Sheppard & H. Cember. 1952. Technique for exposing grasshopper neuroblasts to beta-rays. Nature. **169:** 228.

Rogers, R. W. 1955. α-Particle dosimetry and the inhibition of mitosis in the grasshopper neuroblast by low dosage α-irradiation. Rad. Res. **3:** 18–37.

St. Amand, G. S. & S. R. Tipton. 1954. The separation of neuroblasts and other cells from grasshopper embryos. Science. **119:** 93–94.

St. Amand, W. 1956. Mitotic inhibition and chromosome breakage induced in grasshopper neuroblasts by X-irradiation of known mitotic stages. Rad. Res. **5:** 65–78.

Shaw, E. I. 1956*a*. A glutamic acid-glycine medium for prolonged maintenance of high mitotic activity in grasshopper neuroblasts. Exp. Cell. Res. **11:** 580–586.

Shaw, E. I. 1956*b*. Protection by sodium hydrosulfite against X ray-induced mitotic inhibition in grasshopper neuroblast. Proc. Soc. Exp. Biol. & Med. **92:** 232–236.

DNA SYNTHESIS IN MAMMALIAN KIDNEY CELLS IN TISSUE CULTURE AFTER SINGLE AND PERIODIC DOSES OF IRRADIATION*

Agnes N. Stroud, Austin M. Brues, Beverly R. Svoboda
Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill.

Introduction

Several studies have been made of the ability of cells *in vitro* to synthesize DNA after single doses of irradiation, but little is known about the DNA synthesis of cells after repeated doses. Some investigations have shown that DNA synthesis is inhibited following irradiation;[1-8] others have shown an appreciable increase.[9-12] The present communication will show a marked difference in the ability of two mammalian kidney cell lines from unrelated species and of different chromosome number to utilize tritiated thymidine in the synthesis of DNA, both under normal conditions and following single or periodic exposures to X irradiation. Thymidine uptake was measured in a near-tetraploid rhesus monkey kidney cell line and in a diploid pig kidney cell line.

The tissue culture has certain advantages in tracer studies over whole animal tissue or tissue slices in that it is a preparation of a single cell type, growing as a monolayer on a glass surface; thus experimental interpretation is not subject to the complications that are encountered using a tissue containing many cellular types, possibly of different radiosensitivities. Radiation dosimetry to the cells is more readily calculated under these conditions. Availability of labeled compounds to cells growing *in vitro* is insured, the specific activity of the pool from which a tracer is incorporated is measurable, and unincorporated label is easily accountable by recovery. Cells can be maintained continuously under controlled nutritive conditions, and morphological studies are easily paralleled with tracer studies.

We present data showing that in tissue culture an established monkey kidney cell line utilizes tritiated thymidine at a very low rate, that the rate of uptake is materially increased after irradiation, and that pig kidney cells behave in a very different manner.

Materials and Methods

The monkey kidney cell line (Melnick strain) has been maintained in our laboratory for over 2 years on 70 per cent Morgan's 199 synthetic nutrient, 20 per cent bactotryptosephosphate broth, and 10 per cent calf serum. We have established the fact that the majority of the cells are subtetraploid (FIGURE 1) with the greater percentage in the range of 76 to 78 chromosomes. The pig kidney cell, a diploid line, was obtained 9 mo. ago from Morgan Harris and is maintained on 95 per cent Morgan's 199 synthetic nutrient and 5 per cent calf serum. The chromosome karyotype has remained constant with a stemline number of 37 (FIGURE 1).

* The work described in this article was performed under the auspices of the United States Atomic Energy Commission, Washington, D.C.

The procedures for preparing cultures for the experiments described in this paper were as follows. Monolayers of cells from either strain growing in stock bottles were trypsinized (monkey) or versene-trypsinized (pig) for 15 minutes at 37° C. with a 0.25 per cent solution. The resulting cell suspensions were pooled and centrifuged, the trypsin was decanted, and the cells were resuspended in a known amount of growth nutrient with calf serum to stop the action of the trypsin. An aliquot of the cell suspension was counted with the use of the Coulter counter according to the method of Harris[13] and Peacock,[14] to

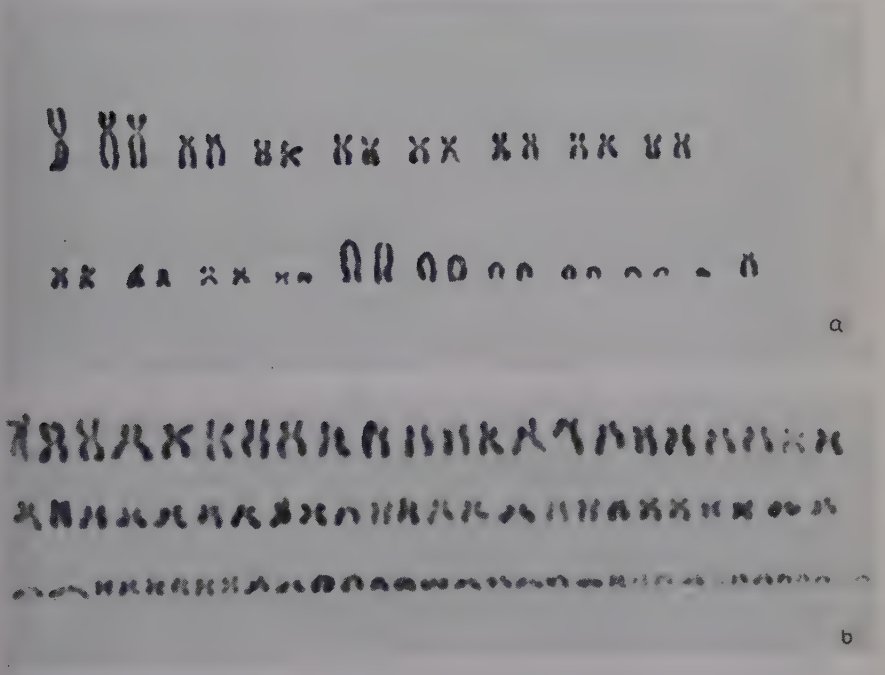


FIGURE 1. (*a*) Chromosome karyotype of pig kidney cells with 37 chromosomes. (*b*) Chromosome karyotype of monkey kidney cells with 78 chromosomes.

estimate the total number of cells, and from this a nutrient solution containing 1 to 2 $\times$ 10^5 cells/ml. was prepared. The culture vessel used throughout the experiments was the Leighton tube; 2 ml. of the stock solution was transferred to each tube. Duplicate or triplicate samples were prepared for each type of observation on each experimental group. For studying the incorporation of tritiated thymidine, Leighton tubes containing glass coverslips (10 $\times$ 35 mm.) were used to facilitate autoradiography. For population studies the same type of tube was used without a coverslip.

Irradiation of the cultures began 18 hours after subcultivation. A 250 kvp.-30 mAmp. X-ray machine with a one-half value layer of 0.9 mm. Cu and a 0.25 mm. Cu and 1.0 mm. Al filter was employed. The tubes with cells and nutrient were placed 65 cm. from the X-ray tube and arranged radially on a

rotating disk. The dose rate in all cases was 100 r/min., and the doses employed were 200, 500 and 1000 r.

In the case of the monkey kidney cells, where repeated radiation was used, cell cultures were permitted to recover from the first X-ray dose; they were then pooled one week after radiation and transferred to stock bottles until repetition of the irradiation three weeks later. Three main experimental

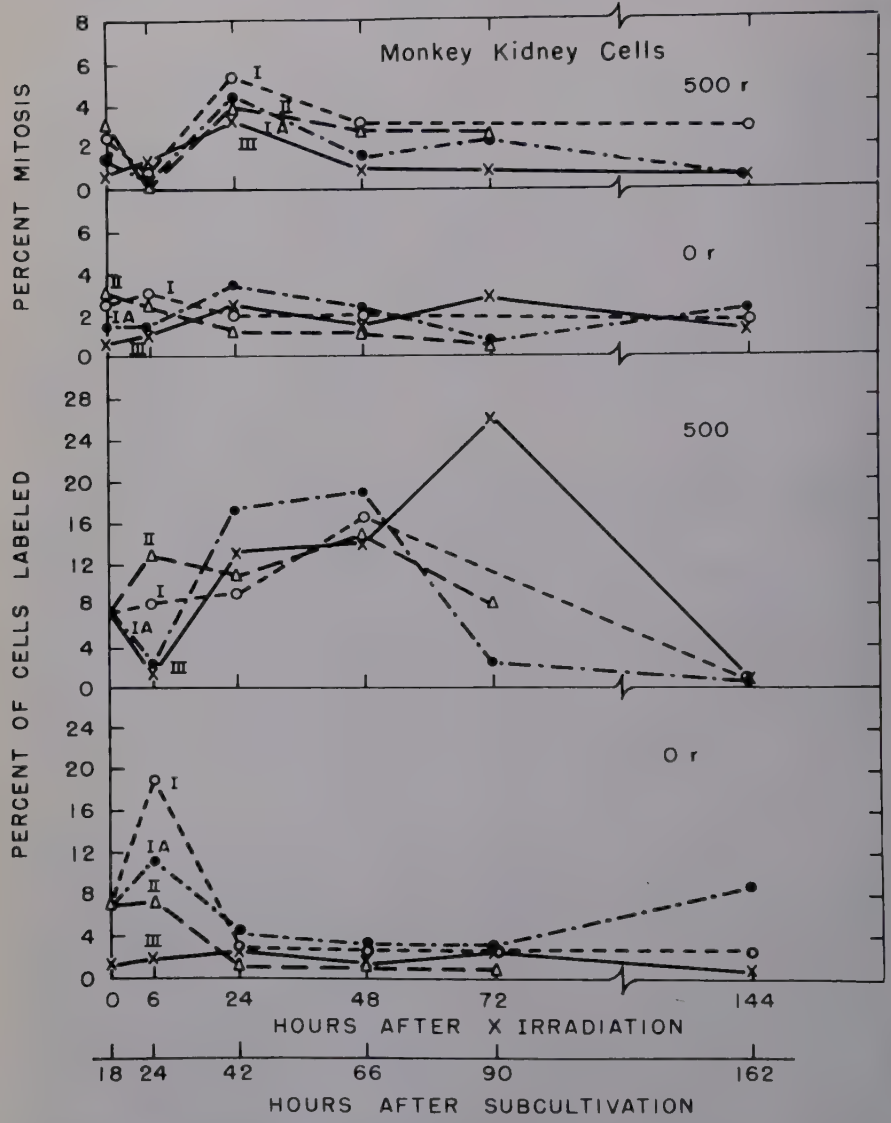


FIGURE 2. Monkey kidney cells: percentage labeled by tritiated thymidine and percentage in mitosis following subcultivation and successive doses of irradiation.

groups are designated as follows: group I consisted of cells given a single dose of irradiation. Four weeks after the first X irradiation the cells were subcultured and transferred to tubes. Group II consisted of cells given a second dose of X ray. Group III consisted of cells established from group II and were given a third dose of X ray. Group IA represented a second single-dose experiment using cells of an unrelated subculture but from the same stem line. The same procedures were followed for the unirradiated control cells.

The tritiated thymidine solution (Schwartz) used throughout the experiment was 1.0 mc./ml. solution with a specific activity of 0.36 c./mM. It was diluted in Morgan's 199 nutrient to the desired concentration. At the specified interval of time after irradiation, tritiated thymidine was added to 2 or 3 tubes of each experimental group to a final concentration of 0.5 μc./ml. These tubes were then incubated for 20 to 30 min. at 37° C., after which the thymidine solution was removed and the coverslips with cells attached were washed with Tyrode's solution, fixed in Carnoy's fluid, and stored in 70 per cent alcohol until the end of the experiment, when all the coverslips were stained with Feulgen. Immediately following staining, Kodak AR-10 stripping film was applied to each coverslip, which had been previously mounted to a glass slide. After 14 days, the films were developed in D19 developer, washed, air-dried, and mounted with Permount on glass slides for microscopic observation.

The number of cells with labeled nuclei above the background of 4 grains was determined from 2 or 3 samples including a total of 500 to 2000 nuclei. From these same samples the number of mitotic figures was recorded and classified according to stages of mitosis. A few grain counts were made on representatively labeled cells in each case.

The total population of cells growing in the Leighton tubes was estimated using the Coulter counter. To carry this out, the nutrient was discarded, 1 ml. of a 0.25 per cent Trypsin or Trypsin-Versene solution in Earle's Saline A was added to each tube, and the tube was incubated at 37° C. for 15 min. The resulting cell suspension was then aspirated back and forth through a small-bore pipette to assure disintegration of any aggregates into a single-cell suspension. Appropriate further dilutions were made with 0.85 per cent saline for cell counting.

The preparation of chromosomes for karyotype analysis was the smear or air-dry method of Hsu (personal communication). A chromosome analysis was made on cells 2 days after they had been subcultured to insure a high mitotic index. Chromosome identification was made on metaphase spreads.

Results

Monkey kidney cells. The extent of the incorporation of tritiated thymidine by nuclei of control and irradiated monkey kidney cells during a period of 8 days after subcultivation and 6 days after 0 r, 500 r, and 1000 r is shown in terms of the mean percentages of cells labeled in TABLE 1 and in FIGURE 2. The data for the nonirradiated monkey kidney cells show an initial high proportion of cells (7 to 19 per cent) incorporating tritiated thymidine 18 to 24 hours after subcultivation (with the exception of group III), followed by a sharp decrease by 42 hours and a leveling to about 4 per cent thereafter. The peak of

incorporation at 24 hours could reflect a synchronous cell population appearing 8 to 10 hours after trypsination and subcultivation. To examine this, hourly determinations of the mitotic indices were made for 10 hours after subcultivation. It was found that within 2 hours the mitotic index dropped to 0.5 per cent or less and, eventually, no mitoses were seen. About 10 hours after subcultivation there was a marked increase in mitotic figures, an indication that the cells were in a phase of synchrony which lasted for about 24 hours. After the initial uptake in all 4 control groups, the cells incorporated tritiated thymi-

TABLE 1

MEAN PERCENTAGE OF MONKEY KIDNEY CELLS LABELED BY TRITIATED THYMIDINE AND PERCENTAGE OF CELLS IN MITOSIS AT VARIOUS TIMES AFTER IRRADIATION

Hours after irradiation	Group I						Group II			
	Per cent labeled			Per cent mitosis			Per cent labeled		Per cent mitosis	
	Control	500 r	1000 r	Control	500 r	1000 r	Control	500 r	Control	500 r
0	7.0	7.0	7.0	2.5	2.5	2.5	7.0	7.0	3.0	3.0
6	19.2	8.3	15.8	3.0	0.8	0.5	7.4	12.8	2.5	<0.1
24	2.7	9.3	0.5	2.0	5.3	1.5	1.1	11.0	1.3	4.0
48	2.8	16.2	0.6	2.0	3.1	7.1	0.9	15.8	1.2	2.9
72	—	—	—	—	—	—	0.5	8.3	1.0	2.8
144	2.6	0.3	<0.1	1.8	3.0	<0.1	—	—	—	—

	Group III				Group IA			
	Per cent labeled		Per cent mitosis		Per cent labeled		Per cent mitosis	
	Control	500 r	Control	500 r	Control	500 r	Control	500 r
0	0.7	0.7	0.7	0.7	—	—	1.5	1.5
6	1.0	1.4	1.0	1.4	11.4	2.6	1.4	0.5
24	12.0	13.3	2.5	3.3	4.3	17.5	3.7	4.3
48	1.0	15.2	1.5	1.0	2.8	19.1	2.1	1.7
72	3.6	26.6	2.9	0.9	2.8	2.6	0.7	2.2
144	0.1	0.8	1.2	0.5	9.2	0.3	2.3	0.4

dine at a surprisingly low rate as compared to the control pig kidney cells (FIGURE 3).

Irradiation of the monkey kidney cells increased the number of cells incorporating tritiated thymidine. After a single or repeated dose of 500 r to the cells, uptake by groups IA and III was decreased at 6 hours after irradiation; this was not seen in groups I and II. In all 4 irradiated groups incorporation was markedly increased at 24 hours and utilization of tritiated thymidine continued at a high level. This high incorporation rate relative to that of the controls persisted for 48 hours after irradiation but fell to control values or less by 72 and 144 hours after irradiation. The delay in the increase in cells incorporating tritiated thymidine in group III (both controls and experimentals) is not understood, but it is noted that mitosis in this group (FIGURE 2) is also retarded suggesting a metabolic delay possibly due to treatment with trypsin, or to other chemical or physical disturbances.

The mean mitotic index for all 4 control groups during the 144-hour experiment was about 2 per cent; however, after repeated doses of 500 r, a sharp decrease to 1 per cent or less was followed by a marked increase of cells in mitosis

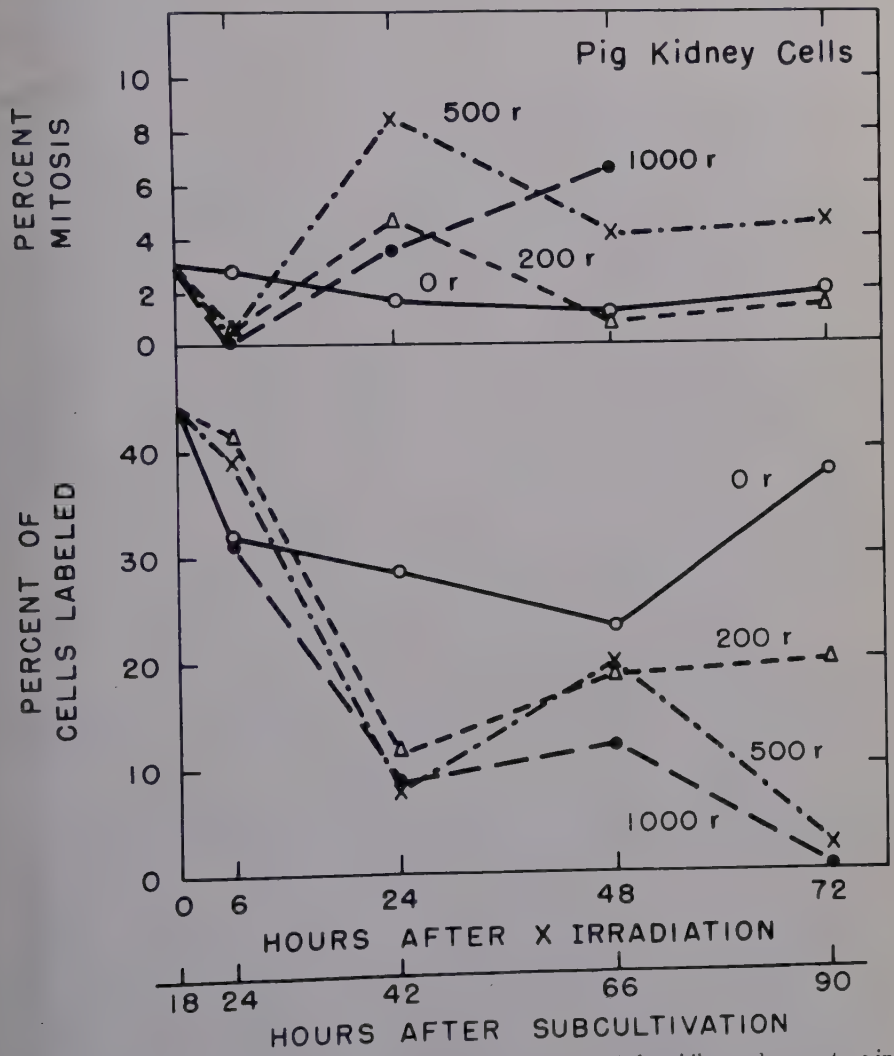


FIGURE 3. Pig kidney cells: percentage labeled by tritiated thymidine and percentage in mitosis following subcultivation and irradiation.

24 and 48 hours after irradiation. This increase represents cells that were delayed in mitosis, and includes a preponderance of abnormal mitotic figures. It is noted that the mitotic index decreases 48 hours after irradiation and that the rate is dependent on the number of doses given.

Pig kidney cells. The incorporation of tritiated thymidine by the pig kidney cells is shown in TABLE 2 and in FIGURE 3, as the mean percentage of labeled

cells after subcultivation and irradiation. Unlike the monkey kidney cells, large numbers of the normal pig kidney cells incorporated tritiated thymidine throughout a 6-day period. The initial high proportion of cells utilizing thymidine in both the controls and the irradiated cells was similar to that observed in the monkey kidney cells. After exposure of the cells to a single dose of 200 r, 500 r, or 1000 r, the incorporation of thymidine decreased to 30 per cent of the control values by 24 hours in all irradiated groups, and did not return to control values even by the 6th day after irradiation.

The mean percentage of pig kidney cells in mitosis after X irradiation is shown in FIGURE 3 and in TABLE 2. An average of 2 per cent of the cells were in mitosis during the 6-day period. As in the monkey kidney cells, irradiation of pig kidney cells resulted in a marked decrease in mitosis to less than 1 per cent in all 3 groups at 6 hours after irradiation, followed by a sharp increase noted at 24 hours. The maximum increase after 500 r was seen 24 hours after

TABLE 2
MEAN PERCENTAGE OF PIG KIDNEY CELLS LABELED AND PERCENTAGE OF CELLS IN MITOSIS AFTER EXPOSURE TO TRITIATED THYMIDINE AT VARIOUS TIMES AFTER IRRADIATION

Hours after irradiation	Per cent labeled cells				Per cent mitosis			
	Control	200 r	500 r	1000 r	Control	200 r	500 r	1000 r
(Subcultivation*)	32.8	32.8	32.8	32.8	4.9	4.9	4.9	4.9
0	43.6	43.6	43.6	43.6	3.0	3.0	3.0	3.0
6	32.1	40.9	39.2	31.1	2.9	0.6	0.2	<0.1
24	28.6	11.6	8.0	9.5	1.8	4.8	8.6	3.6
48	23.0	17.7	19.5	12.4	1.2	1.0	4.2	6.6
72	41.0	20.0	2.5	0.1	2.0	1.4	4.2	<0.1

* Time of transplantation, 18 hours before irradiation.

irradiation and, after 1000 r, at 48 hours; 72 hours after 1000 r, no mitoses were seen. Many of the mitotic cells were abnormal; a few types are represented in FIGURE 4.

Population Studies

Population growth curves for normal and irradiated pig kidney cells are shown in TABLE 3 and in FIGURE 5, in which the mean cell number for each group is plotted against days after X irradiation. The initial inoculum for each tube was 6×10^3 cells. At the time of irradiation, 2 days after subcultivation, the cell population was 2.4×10^4 cells as determined by the Coulter counter.[13,14] The control cells exhibited a nearly exponential increase for 6 days with a mean doubling time over that period of 1.15 days (27.6 hours). After 6 days the population reached an asymptote. After 200 r there was a slight delay in cell growth during the second day, after which the growth rate was parallel to that of the controls. After 500 r the population decreased gradually beginning 2 days after irradiation, but by the 6th day increase was re-established; during this period inhibition of mitosis appears to play a major

role and cell death a minor one. After 1000 r, a marked decrease in the growth curve began at 2 days after irradiation and continued without recovery. In FIGURE 6 is shown the estimated survival curve based on the assumption that the cell number 6 days after irradiation is an index of the proportion of cells surviving at the time of treatment.

Grain Counts

Approximate grain counts were made, under identical conditions of exposure, in representative cells in most of the experiments. These indicate the rate at which the labeled cells are taking up thymidine in synthesis of DNA. Such counts are necessary in order to detect differences in rate of uptake, since a prolonged synthetic phase would increase the number of cells, proportional to the total, labeled, but would decrease the grain count in these cells.

Unirradiated pig kidney cells showed, at the 6 hour period, average grain counts of about 50; at 24 hours, about 100; and at 48 hours, about 35. These are substantially higher than any counts made on the monkey kidney which, if conditions were identical, might have been expected to have higher grain counts per cell due to higher DNA content. Unirradiated monkey kidney cells showed counts between 10 and 20 at all time intervals; after 100 r, about the same; after 500 r, 20 to 30 at 24 and 48 hours, 12 to 15 at 6 hours and, after 1000 r, 15 at 6 and 24 hours. It seems clear from this that grain counts ran parallel to percentages of labeled cells, while if the percentages had reflected different rates of synthesis alone, the grain counts would have been inversely related to percentages of labeled cells.

Chromosome Karyotypes

Monkey kidney cells that had recovered from three repeated doses of 500 r were cloned one month after the third dose of radiation. A few of the clones were isolated for study of their chromosome karyotypes, taken at metaphase, by the chromosome smear technique (Hsu, personal communication). Among the abnormalities were chromosome breaks, deletions, chromatid exchanges, chromosome exchanges and minute fragments. A few examples are shown in FIGURE 7. A chromosome marker with chromatids from a non-homologous chromosome (FIGURE 7*b*) was seen in many of the cells from the same clone, indicating persistence and duplication of this type of abnormality through many cell divisions. The time at which this and other abnormalities arose is not known since the cells were studied only at 1 mo. after the third dose of irradiation.

Pig kidney cells were cloned one month after a single dose of 500 r. Chromatid breaks, inversions, deletions, isochromatid exchanges, chromatid exchanges, chromosome exchanges, and other abnormalities were seen. A few are shown in FIGURE 8. Radiation damage appeared to be more severe after 1 single dose of 500 r than that seen in the monkey kidney cells after 3 doses of 500 r given 1 month apart. In 1 pig kidney cell as many as 2 single chromatid breaks, a chromatid exchange, a chromosome exchange and deletions were observed. Such abnormalities were most often seen in cells which had become polyploid. The occurrence of a chromosome with chromatids from a non-

b
d

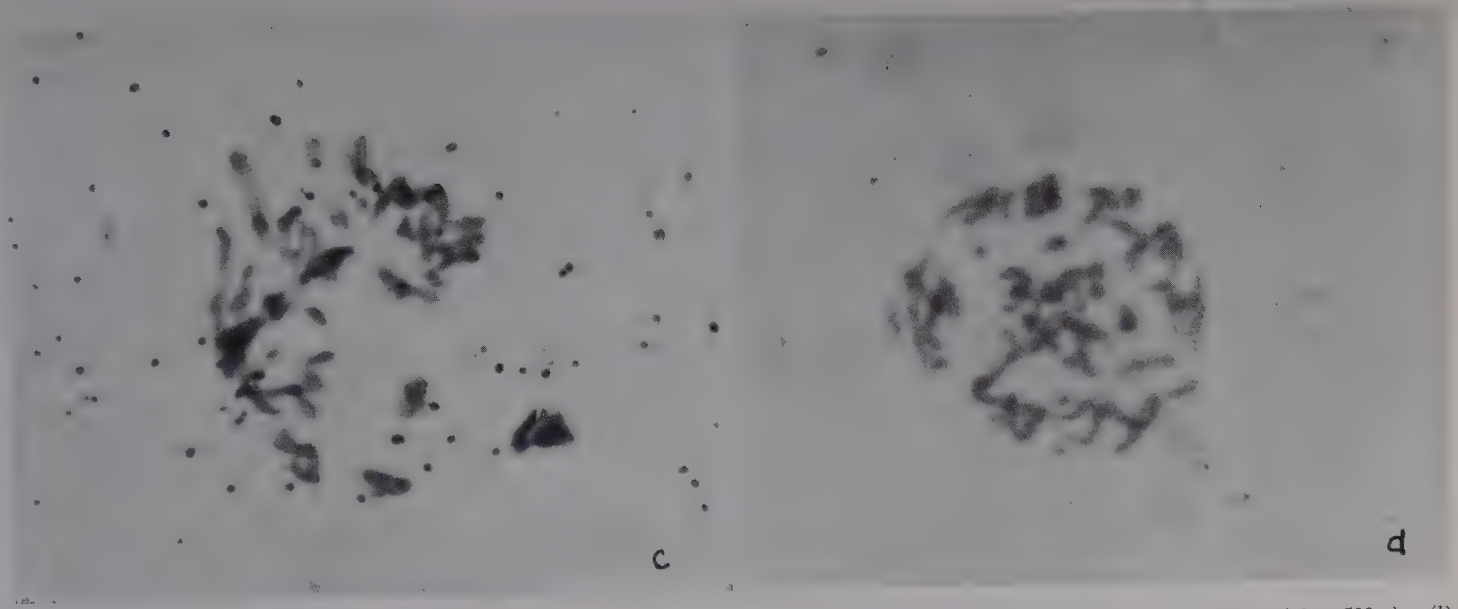


FIGURE 4. Abnormal mitotic figures of pig kidney cells 24 to 48 hours after X irradiation. (*a*) Loss of chromosomes at anaphase (after 500 r). (*b*) Chromosome bridge and loss of chromosomes at anaphase (after 500 r). (*c*) Unorganized metaphase plate with chromosome exchanges (after 1000 r). (*d*) Prophase with permanent or temporary exchange (after 1000 r).

TABLE 3
TOTAL NUMBER OF CELLS IN PIG KIDNEY CULTURES FOLLOWING IRRADIATION
(MEAN OF THREE CULTURES)

Days after X ray	Cells × 10^5			
	0 r	200 r	500 r	1000 r
(Subcultivation*)	0.06	0.06	0.06	0.06
0	0.24	0.24	0.24	0.24
1	0.60	0.60	0.61	0.66
2	1.10	0.87	0.76	0.59
3	1.92	1.45	0.70	0.47
4	3.99	2.22	0.54	0.18
6	7.26	6.64	1.72	0.25
12	16.50	15.70	6.22	0.23
14	17.60	14.30	7.64	0.17

* Eighteen hours before irradiation.

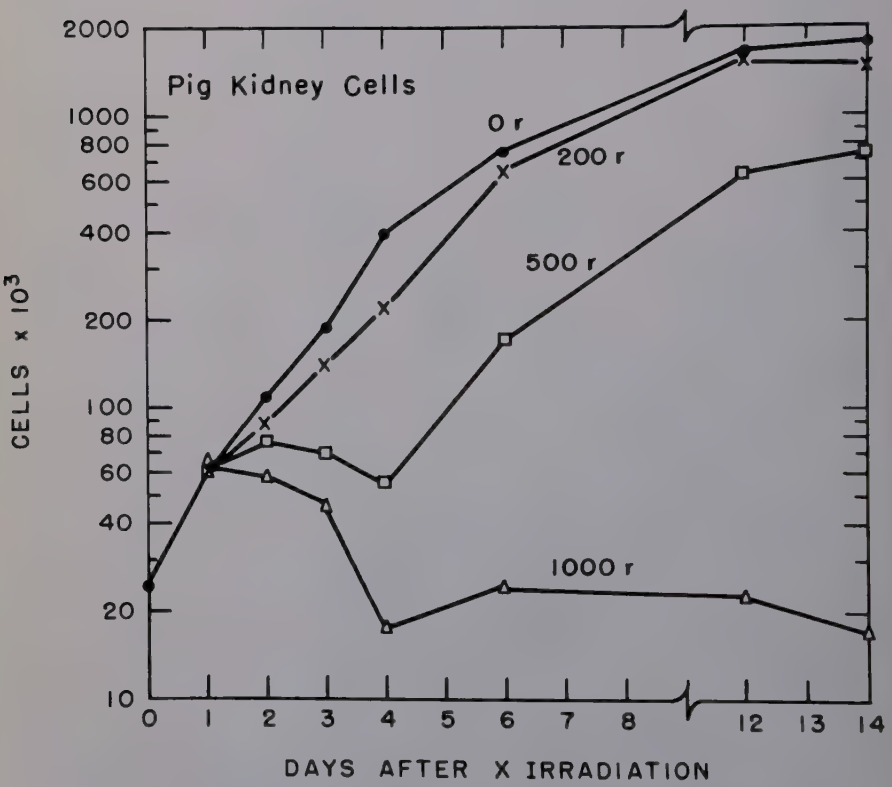


FIGURE 5. Growth of pig kidney cells after subcultivation and irradiation.

homologous chromosome similar to that seen in the monkey kidney cells was persistent in diploid cells and was isolated from one of the clones whose mitotic cells carried the variant. This type of marker was described by Ruddle[15] in

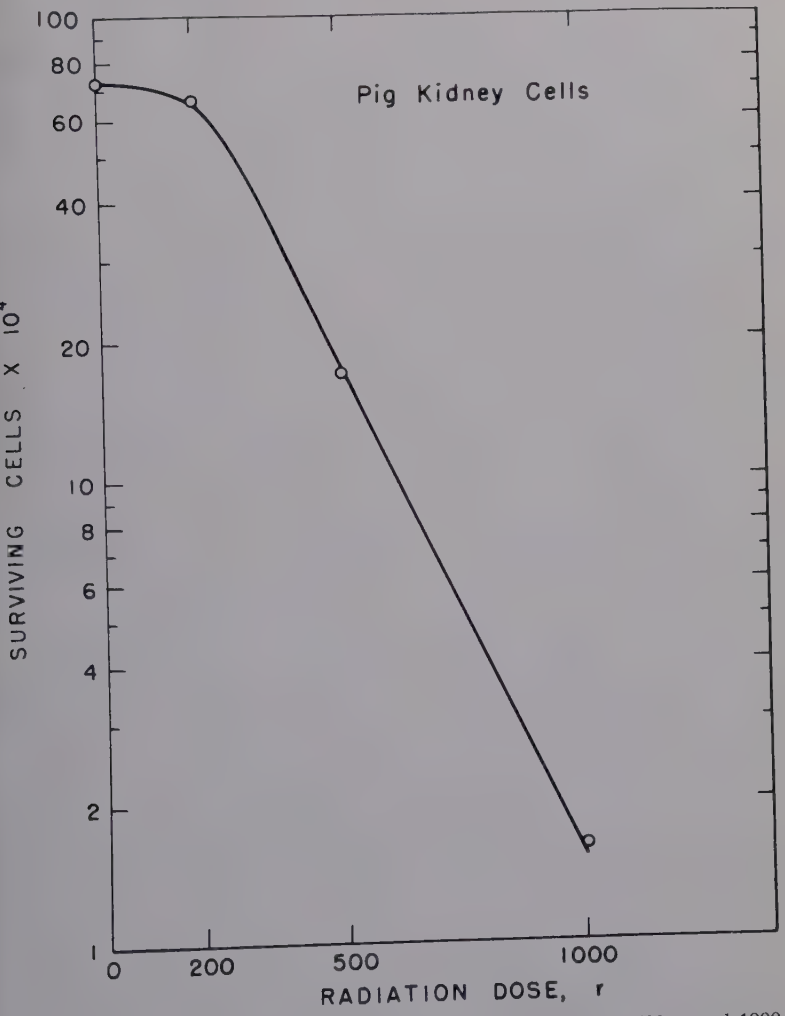


FIGURE 6. Survival curve for pig kidney cells 6 days after 200 r, 500 r, and 1000 r.

the same cell line after sublethal doses of irradiation. Photomicrographs of abnormal mitotic cells, 24 hours after 500 r, Feulgen-stained and not squashed, are presented in FIGURE 4. The occurrence of anaphase bridges with loss of chromosomes was a common occurrence (FIGURE 4*a* and *b*). Many abnormal metaphase structures appeared as well (FIGURE 4*c* and *d*).

Discussion

The difference in the ability of two types of mammalian kidney cell growing *in vitro* to incorporate tritiated thymidine under the conditions of these experiments might be explained on the basis of a biochemical difference. The normal

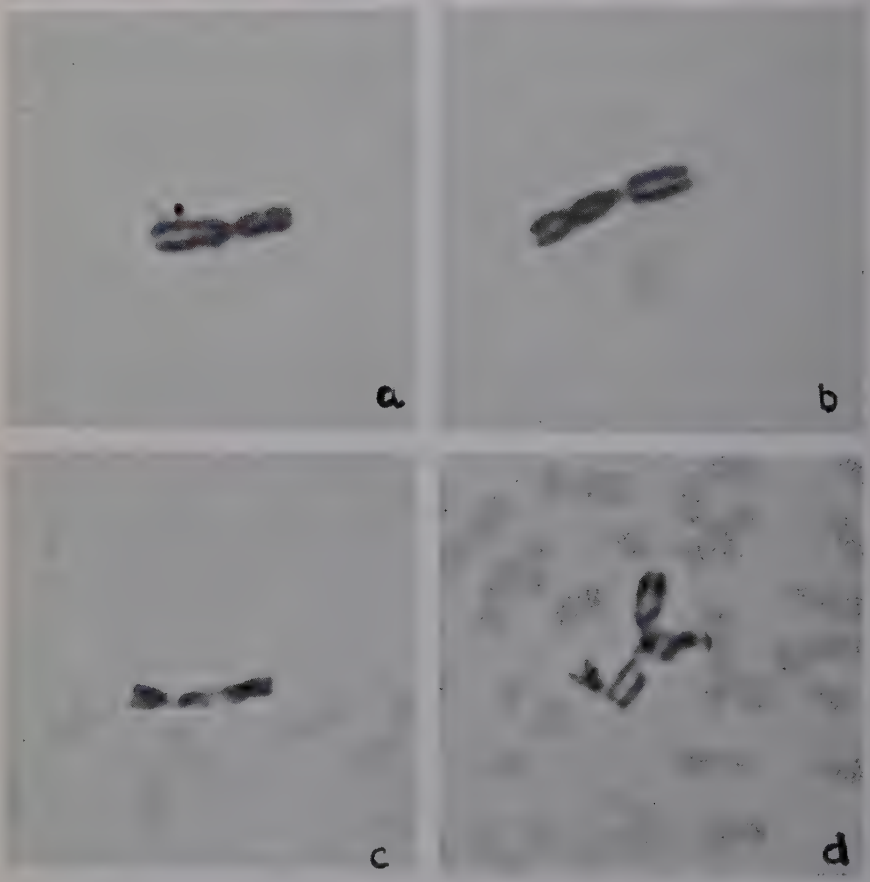


FIGURE 7. Abnormal chromosomes of monkey kidney cells 1 mo. after the third consecutive monthly dose of 500 r. Chromosome squash at metaphase. (*a*) Chromatid break from clone 1. (*b*) Chromatid exchanges with nonhomologous chromatids from clone 1. (*c*) Chromosome break of a chromatid exchange with nonhomologous chromatids from clone 2. (*d*) Chromatid break from clone 2.

monkey kidney cells appear to use a different metabolic pathway for DNA synthesis from that in normal pig kidney cells since relatively few of them utilize thymidine during DNA synthesis. It is possible that they preferentially use thymine or other precursors in the nutrient for DNA synthesis. The fact that after 3 doses of 500 r the irradiated monkey kidney cells incorporated tritiated thymidine, whereas only a small proportion of the control cells utilize it, indicates that the irradiated cells are using exogenous thymidine for DNA

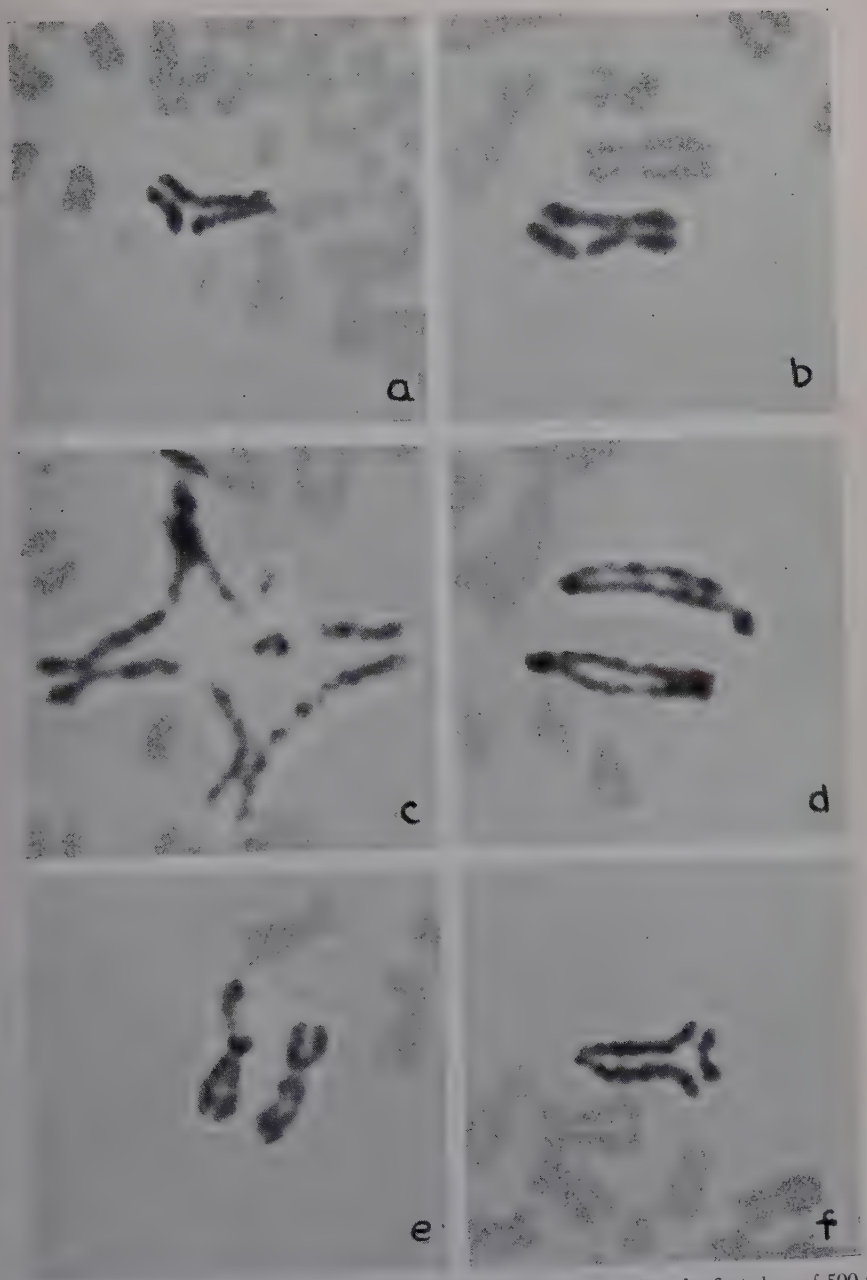


FIGURE 8. Abnormal chromosomes of pig kidney cells 1 mo. after the first dose of 500 r; squash preparation at metaphase. (*a*) Chromosome break of a chromatid exchange with nonhomologous chromatids. (*b*) Chromatid break. (*c*) Chromatid exchanges. (*d*) Chromosome exchange resulting in two dicentrics. (*e*) Chromatid deletion. (*f*) Isochromatid exchange.

synthesis. If this is so there must be a blocking of some key enzyme system necessary for *de novo* synthesis of DNA after irradiation. It is possible that the initial increase in thymidine incorporation by the normal monkey kidney cells may be dependent on a shifting of the intermediary pathway for *de novo* synthesis of DNA, due to metabolic disturbances during subcultivation and followed by synchrony of the cells after attaching to the glass surface. Later, the cells may show a decrease in uptake due both to a reversion to *de novo* DNA synthesis and to loss of the synchrony. Painter and Robertson[10] reported an increase above the control level in the labeling of HeLa S3 cells during the first few hours after irradiation, followed by a fall in the percentage of labeled cells below normal 24 hours after irradiation.

Experiments were conducted to determine whether the concentration of tritiated thymidine (0.5 μc./ml.), the specific activity of 0.36 c./m*M*, or the length of time the tritiated thymidine solution remained on the cells, were sufficient to label properly normal cell nuclei. Exposure of the cells to 1 μc./ml. (3.6 c./millimole) in Morgan's 199 nutrient for 1½ hours (10 times that used originally), resulted in no change in the uptake of the label. Likewise, if the labeled thymidine were diluted in the complete basic nutrient or in Eagle's nutrient, which contains no thymine, there was no difference in labeling. To ensure that removal of the unlabeled nutrient and addition of fresh nutrient with label did not alter the uptake of the label, concentrated or partially concentrated tritiated thymidine (0.5 μc./ml.) was added directly to the unlabeled nutrient; no change in incorporation was observed.

In contrast to the monkey kidney cells, the normal pig kidney cells incorporated labeled thymidine at a level of 30 to 40 per cent, as would be expected at the concentrations and specific activity employed. However, the irradiated cells showed a high uptake of thymidine incorporation only initially, a fact that could also be explained on the basis of cells in synchrony after subcultivation. The marked reduction of mitosis 6 hours after irradiation was not paralleled by a reduction in the number of cells labeled at this time, but was reflected 24 hours after irradiation, when the number of cells labeled in any irradiation group fell to one third of the controls. Mitotic figures increased markedly at 24 hours after irradiation, as a result of delay of cells in mitosis due to radiation damage.[2,16-18] There was no reduction in the growth curve 24 hours after irradiation (FIGURE 5); the doubling time (18 hours) for all the irradiated cells was the same as for the controls during this time, indicating that the cells were able to go through one division, regardless of the stage in the mitotic cycle in which they were irradiated. Injury to the mitotic apparatus of the dividing cells becomes reflected in the growth curves 48 hours after irradiation, which were reduced in all 3 irradiated groups.

Cells recovering from the highest dose of irradiation were subcultured for further study. After a single dose of 1000 r to the monkey kidney cells the incorporation rate of the label was 15 per cent at 6 hours, and dropped off rapidly, but the culture recovered to yield cells for subculturing again at one month. Then, when a second dose of 1000 r was given, the incorporation rate was again high and dropped sharply, but after 2 days the cells were too few to count and, 2 to 3 weeks later, there were no survivors. In the case of the pig

kidney cells we were unable to give a second dose of 1000 r as there were no survivors 4 weeks after the first irradiation. Also, it is occasionally impossible to carry survivors through a second subcultivation and irradiation after a single dose of 500 r. It appears that the pig kidney cells are more radiosensitive than the monkey kidney cells, although the decrease in cell number in cultures that have resumed growth after a few days suggests that the LD_{50} is about the same for the 2 cell types. The greater sensitivity of the pig cells is supported by the fact that they exhibit a larger number of such results as chromatid breaks, deletions, and isochromatid exchanges at 1 mo. after a single dose of 500 r. Very few of these abnormal mitotic figures are observed even after 3 doses of 500 r to the monkey kidney cells. Whether this may be due in part to recovery, by elimination of cells damaged by the first irradiation, remains to be verified. It seems possible that this process might be facilitated in the monkey cells, which have a larger complement of chromosomes.

It is conceivable that variations in the proportion of labeled cells might indicate differences in the rate of DNA synthesis, in which case a higher percentage of labeled cells might indicate a slower buildup of DNA during the synthetic phase, which was thereby prolonged. This seems to be negated by the fact that grain counts ran parallel to the proportion of cells labeled, being higher in the case of the pig kidney cells, lower in the unirradiated monkey kidney cells, and higher in the latter after irradiation.

The product of the grain counts by the proportion of labeled cells, indicating the rate of average synthesis of DNA per cell in the cultures, ranged from 7.4 to 27.6 in the unirradiated pig cells; 2.3 at 6 hours and 0.51 or less at 24 hours and, later, in unirradiated monkey cells; and 2.6 to 5.3 at the 24 and 48 hour periods after irradiation of these cells at 500 r.

One possible difference between diploid and tetraploid cells should be mentioned. Hauschka *et al.*[19] have shown that a tetraploid ascites tumor grows, in its later stages, at about one half the rate of the corresponding diploid, since the growth is limited by the mass of tumor rather than by the cell number, and that the duration of mitosis was prolonged as well as the duration of the intermitotic period; while this may also be true under the conditions of tissue culture, it can hardly account for the decreased rate of synthesis, both by individual cells and on the basis of the number of cells synthesizing DNA at a given time.

For normal growth, the monkey kidney cells, unlike the pig kidney cells, require the addition of bacto-tryptose phosphate to its growth medium. The possibility that this material may either be protective against radiation or that it may be required for recovery of irradiated cells might explain the difference in response of the two types of cells, and this is now being investigated.

Summary

The effect of irradiation upon the uptake of tritiated thymidine by cells *in vitro* has been investigated, employing a pig kidney cell and a monkey kidney cell strain.

The two strains exhibit marked differences in thymidine uptake both in the nonirradiated state and after irradiation. Thirty to 40 per cent of the pig cells incorporate thymidine and this rate of incorporation continues for several

days after subcultivation; after irradiation, the extent of thymidine incorporation is reduced in relation to the X-ray dose (between 200 and 1000 r). Only a small percentage of monkey cells incorporates thymidine except for a period in the first day of subcultivation which may represent a period of synchrony or of recovery from injury of handling; on the other hand, these cells are proliferating at about the same rate as the pig cells. When the monkey cells are irradiated, they show a sharp increase in the percentage of labeling, which continues for several days. It is suggested that normal DNA synthesis in the monkey cells involves some alternate pathway of DNA synthesis not utilizing exogenous thymidine, and that a radiation-induced block of that pathway results in utilization of the labeled thymidine by the cells.

The pig cells are diploid and the monkey cells are subtetraploid. The pig cells are more radiosensitive, perhaps on this account; cultures of these cells fail to grow after a second irradiation with 500 r while the monkey cells grow well after 3 such irradiations. Alternative explanations of the differences in radiosensitivity are under consideration.

References

1. Lajtha, L. G., R. Oliver, T. Kumatori & F. Ellis. 1958. On the mechanism of radiation effect on DNA synthesis. Radiation Research. **8:** 1–16.
2. Harrington, H. 1960. Effect of irradiation on cell division and nucleic acid synthesis in strain U-12 fibroblasts. Biochim. Biophys. Acta. **41:** 461–469.
3. Ord, M. G. & L. A. Stocken. 1957. The effect of X-radiation on rat thymus nucleic acids at short intervals after exposure *in vivo*. *In* Advances in Radiobiology. G. de Hevesy, A. Forssberg & J. Abbatt, Eds. Oliver & Boyd. Edinburgh, Scotland. **:** 65.
4. Holmes, B. E. 1956. Influence of radiation on metabolism of regenerating rat liver. Ciba Foundation Symposium, Ionizing Radiations and Cell Metabolism. Churchill. London, England. **:** 225–226.
5. Lesher, S., A. N. Stroud & A. M. Breus. 1960. The effects of chronic irradiation on DNA synthesis in regenerating mouse liver. Cancer Research. **20:** 1341–1346.
6. Pelc, S. R. & A. Howard. 1955. Effects of various doses of X-rays on the number of cells synthesizing deoxyribonucleic acid. Radiation Research. **3:** 135–142.
7. Kelly, L. S. 1957. Effect of radiation on DNA synthesis in mammalian cells. "Progress in Biophys." Pergamon. New York, N.Y. **8:** 143–163.
8. Looney, W. B., R. C. Campbell & B. E. Holmes. 1960. The effect of irradiation on the replication of deoxyribonucleic acid in hepatocytes. Proc. Natl. Acad. Sci. **46:** 698–708.
9. Painter, R. B. 1960. Nucleic acid metabolism in HeLa S3 cells after X-ray induced mitotic delay. Radiation Research. **13:** 726–736.
10. Painter, R. B. & J. S. Robertson. 1959. Effect of irradiation and theory of role of mitotic delay on the time course of labeling of HeLa S3 cells with tritiated thymidine. Radiation Research. **11:** 206–217.
11. Stroud, A. N., A. M. Brues & B. R. Svoboda. 1959. DNA and protein content of Krebs-2 mouse ascites tumor cells grown under heterologous conditions. Proc. Am. Assoc. Cancer Research. **3:** 67.
12. Whitfield, J. F. & R. H. Rixon. 1959. Effects of X-radiation on multiplication and nucleic acid synthesis in cultures of L-strain mouse cells. Exptl. Cell Research. **18:** 126–137.
13. Harris, M. 1959. Growth measurements on monolayer cultures with an electronic cell counter. Cancer Research. **19:** 1020–1024.
14. Peacock, A. C., G. Z. Williams & H. F. Mengoli. 1960. Rapid electronic measurement of cell volume and distribution. J. Natl. Cancer Inst. **25:** 63–74.
15. Ruddle, F. H. 1960. Chromosome variation in clonal lines of tissue culture cells derived from pig kidney. Radiation Research. **12:** 468.
16. Stroud, A. N. & A. M. Brues. 1954. Radiation effects in tissue culture. Texas Repts. Biol. Med. **12:** 931–944.

17. Stroud, A. N. 1956. The effects of continuous irradiation by tritium on cells cultivated *in vitro*. Ann. N. Y. Acad. Sci. **67:** 11–34.
18. Pomerat, C. M., M. V. Fernandes, Y. H. Nakanishi & S. P. Kent. 1958. Irradiation of cells in tissue culture. V. The effect of gamma irradiation from a cobalt^{60} source on human amnion cells *in vitro*. Zeitschrift für Zellforschung. **48:** 1–9.
19. Hauschka, T. S., S. T. Grinnell, L. Revesz & G. Klein. 1957. Quantitative studies on the multiplication of neoplastic cells *in vivo*. IV. Influence of doubled chromosome number on growth rate and final population size. J. Natl. Cancer Inst. **19:** 13–32.

NUCLEIC ACID METABOLISM AND THE LETHAL EFFECT OF RADIATION ON CULTURED HUMAN CELLS (HeLa)*

Robert B. Painter
Battelle Memorial Institute, Columbus, Ohio

Walter L. Hughes
Medical Department, Brookhaven National Laboratory, Upton, N.Y.

Introduction

Many studies, using a variety of cellular systems, have been made on the effects of ionizing radiation on nucleic acid, particularly deoxyribonucleic acid (DNA) metabolism. Much of the impetus for this work has resulted from the concept that, since DNA is (probably) the genetic material, the primary lesion resulting in cell death might be a result of a defect in DNA metabolism. Results in whole animals have always been confused by (at least the possibility of) differential radiosensitivity of different cell types. Cell cultures offer the advantage of working with "pure cell" lines, in which the population can be considered to be practically homogeneous.

We have been using tritiated precursors of the nucleic acids and autoradiography to study the effects of X radiation on nucleic acid metabolism in HeLa S3 cultures, and attempting to correlate these effects with the lethal ones. Very early in our investigation we noted a toxic effect of tritiated thymidine (H^3TDR)[1] on HeLa cell growth and Ruth Drew, at Brookhaven, has pursued this work.[2] However, we have found that concentrations of 0.05 μc./ml. (3 c./m*M*) or less H^3TDR in the culture medium for periods of no more than one-half hour do not measurably disturb the viability, generation time, or rate of entry into mitosis of the cells. Therefore the use of H^3TDR at these concentrations appears to be a satisfactory tracer for DNA metabolic studies. Tritium-labeled cytidine (H^3CR) has been found to be tolerated in much higher doses than H^3TDR and has been used at a concentration of 1 μc./ml. (1 c./m*M*) as a tracer for RNA metabolism, providing that some provision is made for the DNA labeling such as the use of deoxyribonuclease before the application of the photographic emulsion.

Methods

In the experiments reported here, about 1 ml. of a suspension of HeLa S3 cells in fetal calf serum supplemented Eagle's medium, at a concentration of 3 to 5 $\times$ 10^4 cells/ml., was added to each of several cover slips containing Leighton tubes that were incubated for 2 to 4 days before use. In experiments designed to determine the cells in DNA synthesis at any one time, the H^3TDR was added for the 20 to 30 min. just prior to fixation. In experiments designed to determine the fate of labeled cells at different times following the

* The work described in this paper performed at the Battelle Memorial Institute was supported by Grant No. E-185 from the American Cancer Society, New York, N.Y., and by Contract AT(30-1)2244 with the United States Atomic Energy Commission, Washington, D. C.; the work performed at Brookhaven National Laboratory was supported by the United States Atomic Energy Commission.

labeling period, the HT^3DR containing medium was removed from the tubes, the cells washed 2 times with Hanks balanced salt solution, and a new medium, containing 10 μg./ml. nonradioactive thymidine was added. At the time of sacrifice, medium was removed from the tubes, the cover slips washed with phosphate buffered saline, and acetic-alcohol (1:3) added for 10 to 20 min., after which it was removed and replaced by 70 per cent alcohol. When all the tubes for any one experiment were ready, the cover slips were removed, in most cases stained by the Feulgen method, mounted with HSR onto 1 × 3 microscope slides, dried, and stripping film autoradiograms were prepared. After exposure of the films, which varied from 2 days to 3 months depending on the type of experiment, the Feulgen-stained preparations were counterstained with 0.04 per cent crystal violet; previously unstained preparations were stained through the emulsion with Giemsa at *p*H 5.75.

Irradiations were performed with X rays at 250 kvp, 30 mAmp. at Brookhaven National Laboratory, and 250 kvp, 8 mAmp. at Battelle. In both cases external filtration was ½ mm. Cu and 1 mm. Al, and the irradiations were under conditions of minimal scatter. The dose rate varied from 100 r/min. (Brookhaven) to 40 r/min. (Battelle).

Results and Discussion

After determining the normal patterns of DNA metabolism in the HeLa S3 cell,[3,4] we began investigation on the effects of irradiation on the system. Our first observations were surprising, since the most pronounced effect of 500 r of radiation was one causing an *increase* in the percentage of cells labeled by a short (20 to 30 min.) incubation with H^3TDR, at 4 to 8 hours after the irradiation[5] (FIGURE 1). Since grain counts in the irradiated series were only slightly lower (10 to 20 per cent) than controls, it seemed that the piling up in the synthesis phase (S phase) could not be a result of an effect of the rate of DNA synthesis. However, subsequent studies on the specific activity of isolated DNA after irradiation have shown that the inhibition of rate probably can explain most, if not all, of the "S retention" effect. TABLE 1 shows the results of various doses of X ray using different precursors on the specific activity of DNA. In all cases there is an inhibition, but the range is from 13 to 60 per cent. The discrepancy in the results cannot yet be explained, but it is probable that the immediate effect of irradiation on DNA synthesis can be great enough to account for the increase in percentage of cells in DNA synthesis at 6 hours after irradiation. However, it is important to note that the kinetics of DNA synthesis rate inhibition do not follow the same kinetics as cell death, since 500 r kills 90 to 100 per cent of HeLa S3 cells.

The piling up of cells in the S phase also shows that the normal flow of cells into the S compartment from G_1 is unaffected by the X irradiation. This is true up to doses of at least 4000 r (the highest doses we have investigated) and leads to a longer pile up in S (FIGURE 2). The lack of "G_1 effect" is in marked contrast to the findings of Pelc and Howard[6] in *Vicia faba*, of Holmes[7] (first among others) in regenerating liver, and of Lajtha[8] in bone marrow culture. However, at least the results in *Vicia faba* and in bone marrow may be secondary effects of mitotic delay which acts by diminishing the numbers of cells re-

entering the G_1 and subsequently the S phase at relatively long times after the irradiation. The results in regenerating rat liver cannot be due to any such effect; recent results of Van Lancker[9,10] have shown conclusively that the effect is due to a failure of the irradiated cells to polymerize the deoxynucleoside phosphates. Others have failed to find a "G_1 effect,"[11,12] and it is quite possible that this effect in regenerating liver is due to the radiosensitivity of the

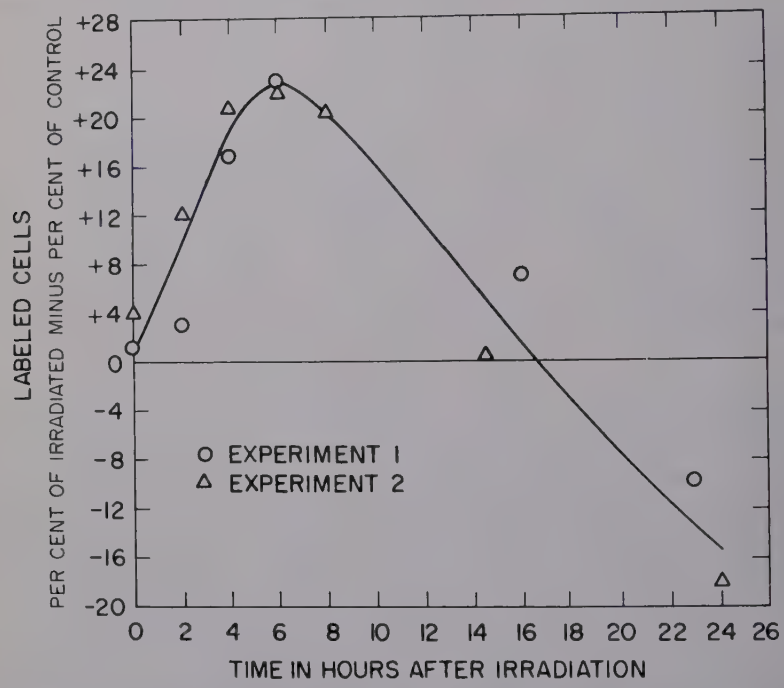


FIGURE 1. Uptake of H^3TDR into Feulgen-positive nuclear material of irradiated HeLa S3 cells. Data plotted are per cent cells labeled by 20-min. incubation with H^3TDR in irradiated population minus the percentage of cells labeled by a similar incubation, at the same time, in controls. Reproduced by permission of *Radiation Research.*

TABLE 1

INHIBITION OF RATE OF DNA SYNTHESIS IN HeLa S3 CELLS BY X RAY: TRACER INCUBATION IN EACH CASE WAS FOR THE FIRST SIX TO SEVEN HOURS AFTER IRRADIATION

Tracer compound	X-ray dose (r)	Average specific activity*		Per cent inhibition
		Control	Irradiated	
H^3-Thymidine	500	2743	1017	63
H^3-Thymidine	500	4415	1691	62
C^{14}-Formate	500	386	336	13
C^{14}-Thymidine and†	1250	3008	1534	49
H^3-Thymidine†	1250	4444	2157	52

* As CPM/UV absorption unit.
† Double tracer experiment.

inducible formation of polymerase, an enzyme which is probably constitutive in cells which are normally undergoing rapid division.

In order to determine more directly if there is a radiosensitive part of the cell cycle, cells were incubated with tritiated thymidine for 30 min., washed, incubated in carrier medium, and irradiated with 1500 r at various times. The Leighton tube cultures were fixed at various times, up to 36 days after the irradiation, and the fraction of labeled surviving giant cells was autoradiographically determined. Cells labeled immediately before or after the irradiation were in the S phase during the irradiation. Cells labeled 5 hours before irradiation were practically all in the post-DNA synthesis, premitotic phase

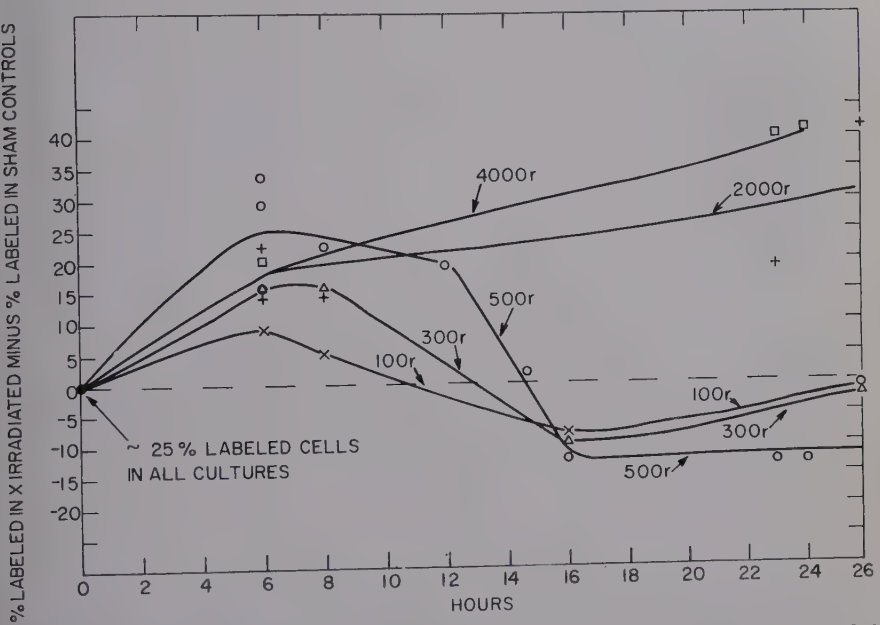


FIGURE 2. The effect of various doses of X radiation on the percentage of cells labeled by a 20-min. incubation with H^3TDR at various times after the irradiations. Data plotted in the same manner as FIGURE 1.

(G_2) at the time of irradiation. Cells labeled 11 to 17 hours before the irradiation were predominantly in G_1 during the irradiation. A typical giant cell, labeled just before irradiation and fixed 14 days later, is shown in FIGURE 3.

The results of these experiments are given in FIGURES 4, 5, and 6, and show that the probability of a cell surviving as a giant is independent of its position in the cell cycle at the time of irradiation. The increase in the percentage of labeled cells (of cells labeled in S, FIGURE 4) is not always observed; however, it has been found at least three times, and is difficult to explain in light of the fact that no significant decrease in the percentage of labeled cells has been observed in cultures of cells labeled in the other phases. It may reflect a tendency for S cells to be more capable of initiating division following irradiation than are G_2 cells.[13] As a result, the cells in S may begin giant cell forma-

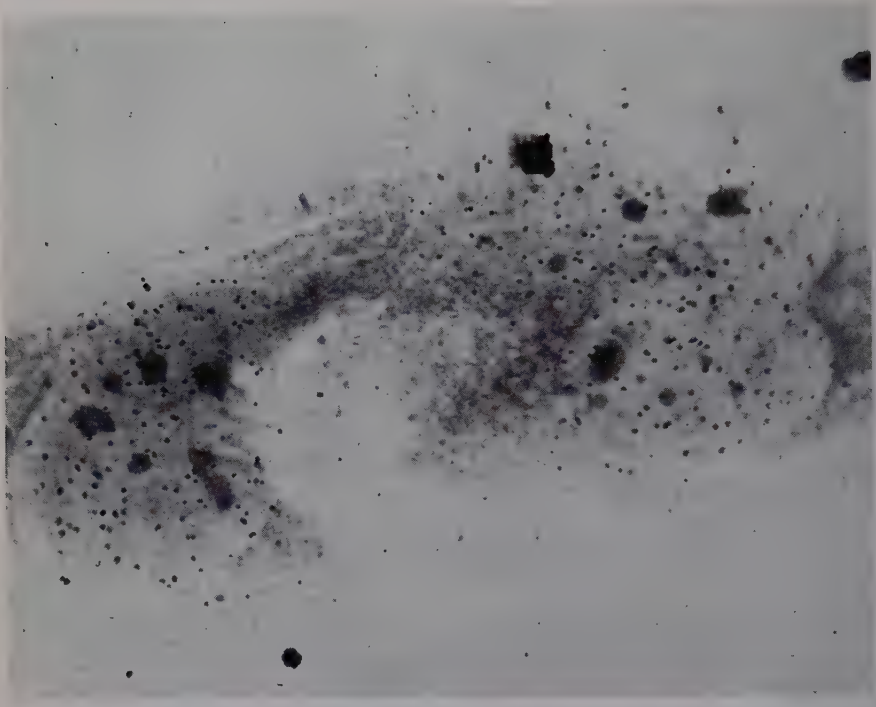


FIGURE 3. Giant cell from a population labeled by a 30-min. incubation with H^3TDR immediately after 1500 r X radiation, and fixed 14 days later. Giemsa, ×900.

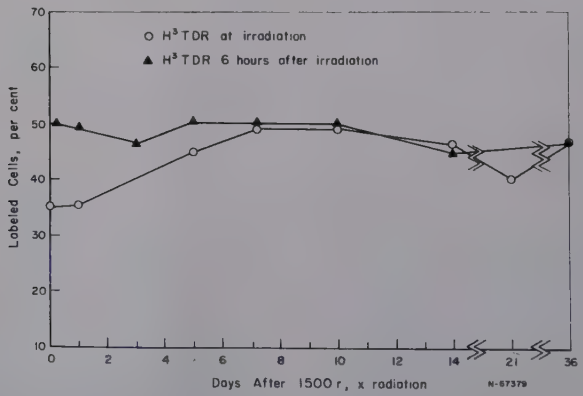


FIGURE 4. Percentage labeled cells in HeLa S3 cultures irradiated with 1500 r and incubated with tracer so that labeled cells were in S phase during irradiation. In one series (○—○), the cultures were incubated with 0.02 μc./ml. tritiated thymidine for the 30 min. immediately following irradiation. In the other (▲—▲) the cultures were incubated with the tracer for 30 min., but the incubation did not begin until 6 hours after the irradiation. Reproduced by permission of *Radiation Research*.

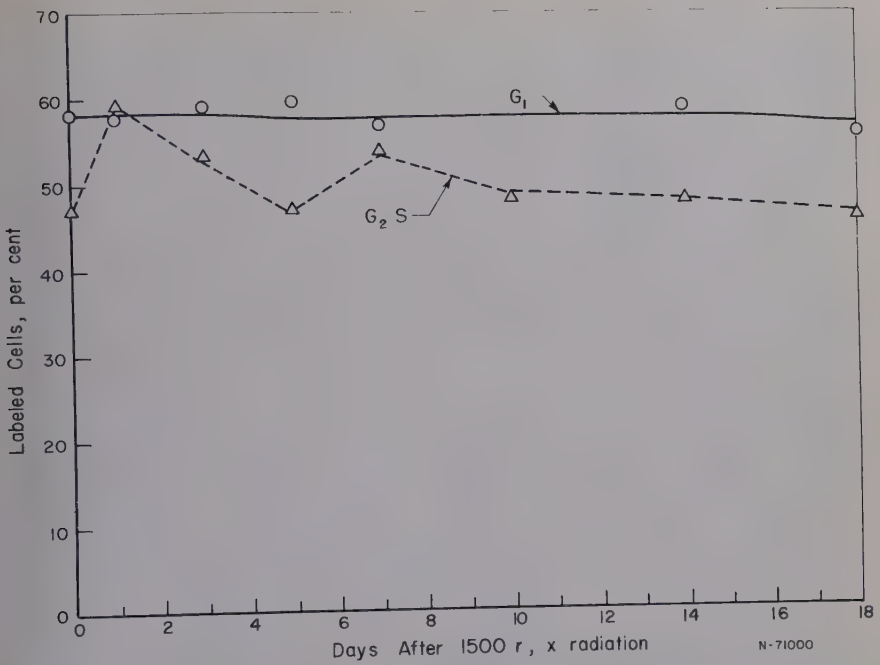


FIGURE 5. Percentage of labeled cells in HeLa S3 cultures irradiated with 1500 r and incubated with tracer so that labeled cells were in G_1 phase and G_2 and S phases during irradiation. The G_1 series was incubated with 0.01 μc./ml. tritiated thymidine for 6 hours, occurring 17 to 11 hours before irradiation. Series G_2S cells were incubated with the tracer for the 6 hours immediately preceding the irradiation. Reproduced by permission of *Radiation Research*.

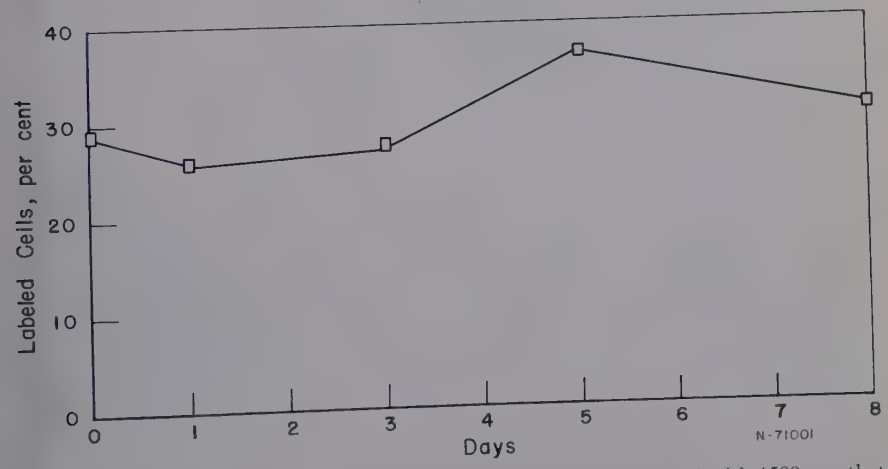


FIGURE 6. Percentage of labeled cells in HeLa S3 cultures irradiated with 1500 r so that labeled cells were in G_2 phase during irradiation. These cells were incubated with tritiated thymidine for a 30-min. incubation that ended 5 hours before the irradiation. Reproduced by permission of *Radiation Research*.

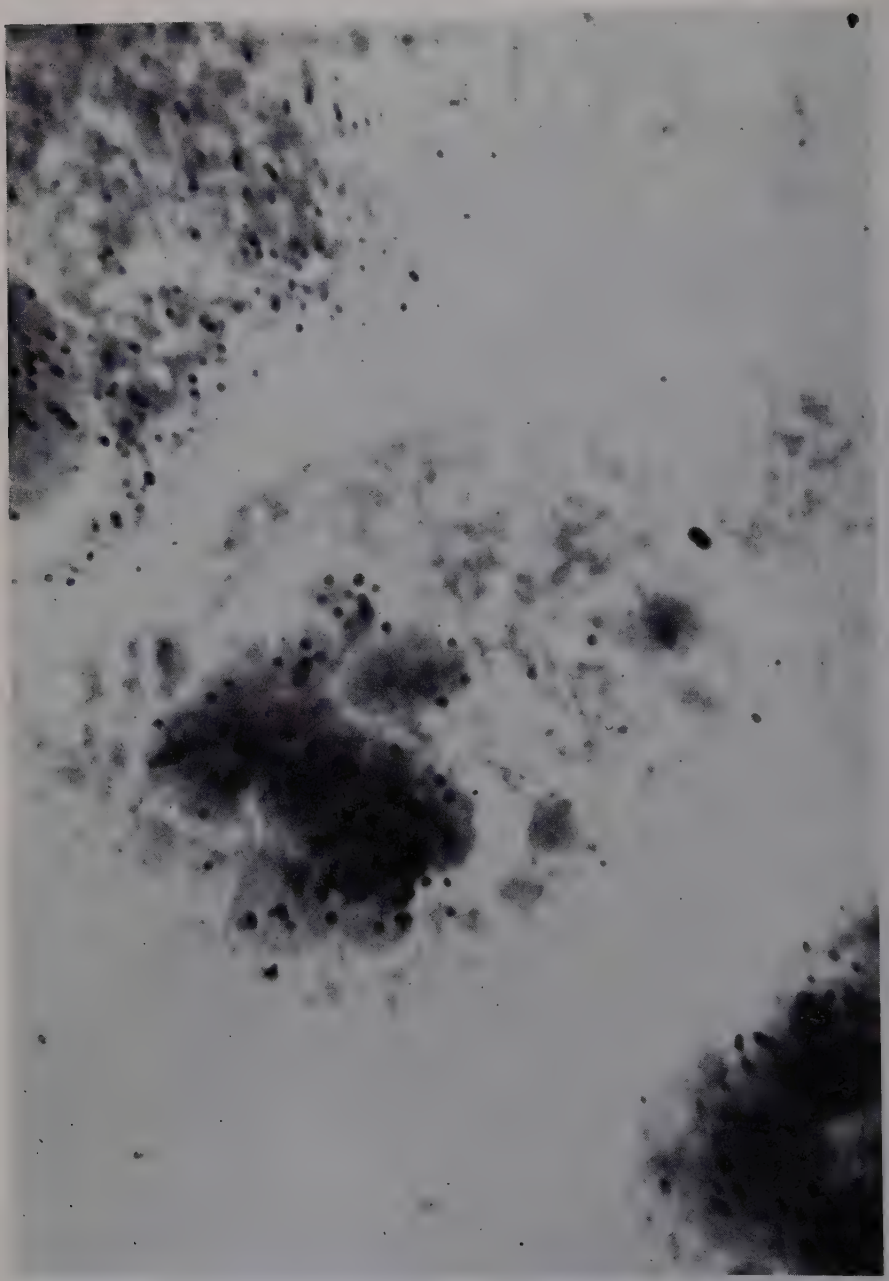


FIGURE 7. From a culture 3 days after irradiation with 1500 r. The culture had received H^3-cytidine 6 hours previously, had been washed, and unlabeled cytidine-containing medium had been added. The great majority of cells showed chromatin, nucleolar, and cytoplasmic labeling at this time. However, the cell with fragmented nucleus in the center of the photograph shows no cytoplasmic labeling as does its neighbor. Reproduced by permission of *Radiation Research*.

tion in proportionally greater numbers in some cultures. In general, however, since the ratio of labeled cells to unlabeled cells in surviving giant cells remains at (or returns to) practically the same level as the original ratio, regardless of their position in the cell cycle at the time of irradiation, the conclusion seems inescapable: there does not appear to be a radiosensitive portion (in terms of viability) of the cell cycle. Therefore, the probability of cell death as a result of irradiation does not appear to be a function of the metabolic state of its DNA. This is surprising, for since the eventual cause of death is thought to be a result of damage to the genetic material, it would be expected that the state of organization of the DNA (for example, replicating or nonreplicating, at the molecular level; in the chromosome or chromatid state, at the organelle level) would have an influence on its susceptibility to irradiation. This does not seem to be the case.

The possibility that giant cell formation may not follow the same pattern as surviving *normally dividing* cells does not appear probable. While our results are not yet complete, the labeled fraction of cells remaining in cultures irradiated with 100 r and 300 r does not appear to change markedly after 3 or 4 generations following resumption of mitosis. These results are somewhat difficult to assess because of the mixtures of dividing and aberrant cells present after irradiation, and because of the longer exposure time of film necessary to obtain good labeling in the cells that have had the tracer diluted by one sixteenth or more as a result of cell division. At the present time it seems likely that the results will correlate well with the giant cell data. If so, it would be necessary to reject the concept of a radiosensitive portion of the cell cycle.

We have also attempted to determine the effects of irradiation on RNA metabolism in the HeLa S3 cell. The normal sequence of events following a short incubation with H^3Cy has been described by Feinendegen *et al.*[14] Thus far the pattern of chromatin labeling followed by nucleolar labeling, followed by cytoplasmic labeling, has not been qualitively altered by doses of X irradiation up to 1500 r and, in giant cells, as late as 14 days after the irradiation, with a rare exception. About 3 days after 1500 r, in populations where many cells exhibit nuclear fragmentation, some of these fail to show cytoplasmic labeling 6 hours after a 30 minute incubation with H^3Cy (FIGURE 7). Since this type of cell largely disappears from the population soon after this time, it is probable that this observation only reflects a general metabolic failure in these cells. However, since the RNA of the nucleus is labeled, and DNA synthesis can occur in such cells,[13] the transfer process of the cytidine-labeled material (presumably RNA) is presumably more influenced by the radiation-induced nuclear disorganization than are the RNA and DNA synthetic processes.

Summary

The results presented here, showing the lack of correspondence of effect of irradiation on DNA synthesis and the lethal effect, and the absence of a radiosensitive part of the cell cycle, as well as the results reported in these pages by others, are very strong evidence that the defect (if any) induced in DNA metabolism has little or nothing to do with the effects causing mitotic delay and death. Moreover, irradiation does not appear to have any effect on the rate

of entry of cells into DNA synthesis, that is, there is no "G_1 effect", and ribonucleic acid metabolism is almost completely unaffected by highly lethal doses of X radiation.

References

1. Painter, R. B., R. M. Drew & W. L. Hughes. 1958. Inhibition of HeLa growth by intranuclear tritium. Science. **127:** 1244.
2. Drew, R. M. & R. B. Painter. 1959. Action of tritiated thymidine on the clonal growth of mammalian cells. Radiation Research. **11:** 535.
3. Painter, R. B. & R. M. Drew. 1959. Studies on deoxyribonucleic acid metabolism in human cancer cell cultures (HeLa). Lab. Invest. **8:** 278.
4. Painter, R. B., R. M. Drew & B. G. Giauque. 1960. Further studies on deoxyribonucleic acid metabolism in mammalian cell cultures. Exptl. Cell Research. **21:** 98.
5. Painter, R. B. & J. S. Robertson. 1959. Effect of irradiation and theory of role of mitotic delay on the time course of labeling of HeLa S3 cells with tritiated thymidine. Radiation Research. **11:** 206.
6. Pelc, S. R. & A. Howard. 1955. Effects of various doses of X-rays on the number of cells synthesizing deoxyribonucleic acid. Radiation Research. **3:** 135.
7. Holmes, B. E. 1956. Influence of radiation on metabolism of regenerating rat liver. Ciba Foundation Symposium, Ionizing Radiations. **:** 225.
8. Lajtha, L. G., R. Oliver, T. Kumatori & F. Ellis. 1958. On the mechanism of radiation effect on DNA synthesis. Radiation Research. **8:** 1.
9. Van Lancker, J. L. 1960. Metabolic alterations after total body doses of X-radiation. I. The role of regenerating liver nuclei and cytoplasm in the inhibition due to X-radiation of incorporation of tritium-labeled thymidine into DNA. Biochim. Biophys. Acta. **45:** 57.
10. Van Lancker, J. L. 1960. Metabolic alterations after total body doses of X-radiation. II. Incorporation of deoxycytidilic and thymidilic acid into purified DNA and nuclei in presence of regenerating-liver supernatant. Biochim. Biophys. Acta. **45:** 63.
11. Caspersson, T., E. Klein & N. R. Ringertz. 1958. Cytochemical studies on some effects of X-radiation on three ascites tumors. Cancer Research. **18:** 857.
12. Harrington, H. 1960. Effect of irradiation on cell division and nucleic acid synthesis in strain U-12 fibroblasts. Biochim. Biophys. Acta. **41:** 461.
13. Painter, R. B. 1960. Nucleic acid metabolism in HeLa S3 cells after X-ray induced mitotic delay. Radiation Research. **13:** 726.
14. Feinendegen, L. E., V. P. Bond, W. W. Shreve & R. B. Painter. 1960. RNA and DNA metabolism in human tissue culture cells studied with tritiated cytidine. Exptl. Cell Research. **19:** 443.

THE INFLUENCE OF INTRANUCLEAR IRRADIATION ON THE GROWTH OF HeLa CELLS IN AGITATED FLUID MEDIUM*

P. C. Sanders, D. F. Petersen, W. H. Langham
Los Alamos Scientific Laboratory, University of California, Los Alamos, N.Mex.

This report outlines the application of several novel techniques that have been utilized to obtain quantitative data on the absolute uptake of thymidine and the influence of incorporated tritium beta irradiation on cellular proliferation in agitated cultures maintained for relatively prolonged periods. Culture of HeLa cells in agitated fluid medium has been coupled with multichannel electronic particle counting and quantitative beta counting procedures that will be outlined in some detail.

Results of these studies confirm and extend previous observations by Painter and his associates[1-4] on effects of beta radiation within a small sensitive volume.

Materials and Methods

Tissue culture. HeLa S3 cells were maintained as stock cultures in T-flasks containing Eagle's basal medium supplemented with 10 per cent horse serum, 0.25 μg./ml. penicillin, and 0.25 μg./ml. streptomycin. Adaptation of glass-grown cells to agitated cultures was accomplished by modifications of McLimans' techniques.[5] Stock cultures were examined under phase contrast and selected on the basis of frequency of mitotic figures. T-Flasks exhibiting a large proportion of cells in active mitosis were trypsinized† for 5 min. at 37° C., counted, and transferred to the spinner flasks. Each flask contained a final volume of 300 ml. of Eagle's basic spinner medium supplemented with 10 per cent horse serum, penicillin, and streptomycin in the same concentrations as for the basal medium. Preliminary investigations demonstrated that optimum growth was attained in cultures containing an initial inoculum of 3.5×10^4 cells/ml. The flasks were gassed with 5 per cent CO_2 in air to *p*H 7.2, and the mixing bar was adjusted to turn at approximately 100 to 120 rpm. Cells started from T-flasks were serially passaged in spinner culture for at least 1 mo. (4 to 5 passages) before use in an experiment.

Cell counting and viability evaluation. Quantitative estimation of the cell population was determined at frequent intervals during growth by removal of small aliquots from the culture medium which, after appropriate dilution, were enumerated in an electronic particle counter‡ equipped with a 100-channel pulse height analyzer. Dilutions in n-saline were adjusted to yield approximately 6 to 10 thousand cells/ml. to ensure accurate counts with threshold settings adjusted to reject particles with volumes less than 2000 μ^3. Size distribution studies conducted on control cultures indicated that these conditions yielded accurate population estimates without interference from smaller debris.[6] Viability of the cells at each interval was judged microscopically

* The work described in this paper was performed under the auspices of the United States Atomic Energy Commission, Washington, D.C.
† Using 1:300 Difco trypsin (0.035 per cent).
‡ Coulter Electronics, Inc., Chicago, Ill.

from the appearance of cell suspensions stained with trypan blue (C. C. Lushbaugh, personal communication). Viability remained from 95 to 98 per cent during the course of these experiments.

Radioactive precursor. The tritiated thymidine used for these studies was synthesized in our laboratory by catalytic exchange over platinum essentially as described by Verly and Hunebelle.[6] The final preparation had a specific activity of 478 mc./m*M* and was chromatographically homogeneous in the presence of authentic carrier thymine and thymidine.

Scintillation counting. A liquid scintillation counting system compatible with aqueous samples has greatly facilitated these studies. The scintillation solution developed in our laboratory by Ott *et al.*[7] consists of 125 gm. naphthalene, 7.5 gm. of recrystallized PPO, 0.375 gm. of POPOP, and dioxane to a final volume of 1 l. With 1-ml. aqueous samples of *p*H 6.5 to 6.8 and 15 ml. of the scintillation solution, counting efficiencies of approximately 20 per cent were obtained. An automatic 2-channel liquid scintillation spectrometer* was used for radioactivity determinations. After replicate counting of the samples, tritium water internal standards were added to determine the extent of quenching. It was found that under the conditions of these experiments, quenching was negligible, and internal standard additions were subsequently abandoned.

Autoradiographic procedures. Cells from a 4.0-ml. aliquot were allowed to settle out, the media removed except for 0.5 ml., resuspended, placed on a slide, and uniformly smeared. The slides were stained by the Feulgen procedure, covered with stripping film† according to the method of Doniach and Pelc,[8] and exposed 3 to 5 days. The autoradiograms were developed, and the preparations were counterstained with 0.004 per cent crystal violet.

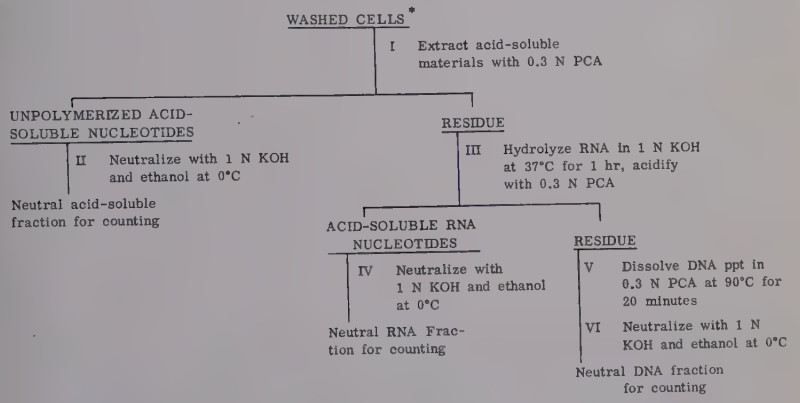
Biochemical procedures. Total thymidine content was determined by suspending an exhaustively washed aliquot of cells in distilled water and subjecting it to sonic disintegration until no further increase in count was obtained. In addition, a greatly simplified chemical separation was devised based on the classical procedures of Schmidt and Thannhauser[9] and Schneider.[10] Details of the procedure are summarized in the flow diagram shown in FIGURE 1. After washing in Hanks' balanced salt solution, centrifuged pellets containing a known number of cells were extracted for 20 min. at 0° with 0.3 *N* perchloric acid (PCA). An aliquot of the extract was removed, and the residue was washed repeatedly with 0.3 *N* PCA at 0° C. The residue was hydrolyzed for 1 hour in 1 *N* KOH at 37° C., acidified with 0.3 *N* PCA, and centrifuged. An aliquot was again removed, and the precipitate was again washed with PCA. The final sediment was hydrolyzed in 0.3 *N* PCA for 20 min. at 90° C. and a third aliquot taken. PCA was removed from each of the 3 aliquots by addition of precisely adjusted amounts of 1 *N* KOH at 0° C., resulting in neutral aqueous solutions representing acid-soluble, RNA, and DNA nucleotides, respectively. One-ml. aliquots of these solutions were then counted in the compatible scintillation system. Since thymidine was the only precursor used in these studies, fractionation of lipids and proteins was omitted but could be included in the procedure in experiments employing other precursors.

* Packard Instrument Company, La Grange, Ill.

† Eastman Kodak stripping film A. R. 10, Eastman Kodak Co., Rochester, N.Y.

Results

Cell size determination. Electronic particle counting enjoys the advantage of providing extremely good counting statistics conveniently. However, routine application of electronic particle counters requires careful cross calibration with visual procedures as well as frequent checks of the instrument with par-



*Cells diluted 5-fold and washed once with Hanks' balanced salt solution

FIGURE 1. Flow diagram for the separation and quantitative determination of radioactivity in cell fractions containing tritiated thymidine.

TABLE 1

EXPERIMENTAL SPINNER FLASKS

Flask No.	Total thymidine content (γ/ml.)	Specific activity H^3-thymidine (mc./mg.)	Specific activity (μc./ml.)
I	40.0	1.98	40.0
II	40.0	0.99	20.0
IV	40.0	0.49	10.0
VI	40.0	0.25	4.0
VIII	40.0	0	0
III	0	0	0

ticles of known size and morphology. Particle sizing of HeLa cells in spinner culture by means of an electronic particle counter and 100-channel pulse height analyzer indicated that the mean volume of the HeLa cell under our culture conditions was 2700 μ^3, in good agreement with published values for these cells under other culture conditions.[11] Visual and electronic estimates of nuclear volume, assuming spherical configuration, ranged from 900 to 1750 μ^3 with a mean volume of 1440 μ^3.

Details of the design of the radiation experiments are shown in TABLE 1. The total thymidine content of the flasks was maintained at a constant level

of 40 μg./ml., while the specific activity was varied by dilution with carrier from 40 to 4 μc./ml. An identical experiment was performed using tritium water in which the specific activity was varied from 90 to 5.4 μc./ml. Data shown in FIGURE 2 demonstrate that radioactivity introduced into actively dividing cultures exerted no immediately discernible effects. Cells in these experiments exhibited generation times of the order of 30 hours regardless of treatment. However, during the interval from 48 to 96 hours after addition of radioactivity in the form of tritiated thymidine, marked effects were noted

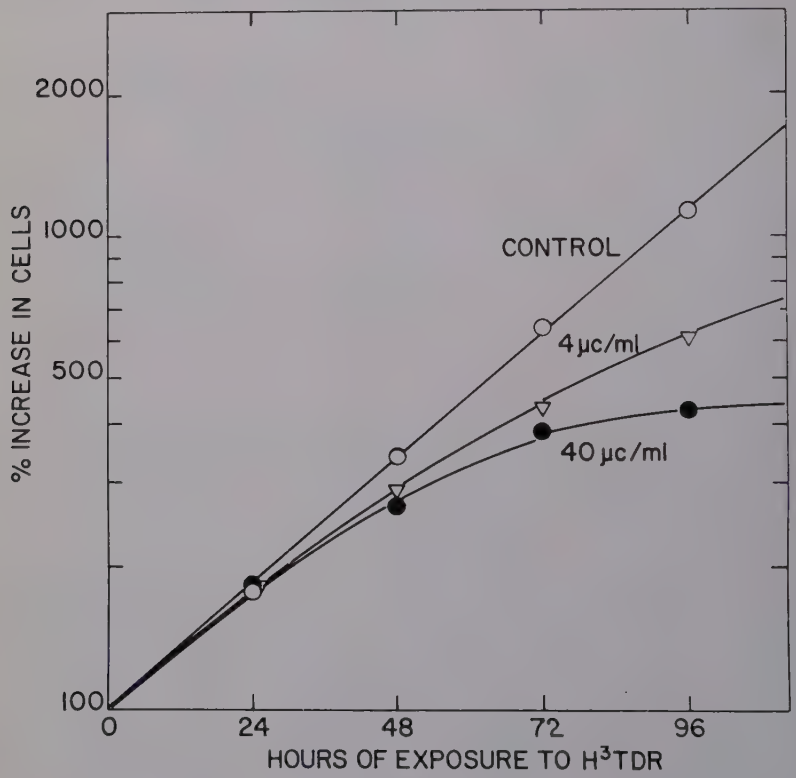


FIGURE 2. The influence of tritiated thymidine on cell proliferation.

which appeared to be directly dependent upon the specific activity of the precursor. Tritium water, on the other hand, at twice the highest specific activity of thymidine, exerted no discernible effect.

Distribution studies performed at the end of one generation time, when all cells were presumed to be labeled but before marked decreases in proliferation occurred, indicated that approximately 90 per cent of the label was fixed in deoxyribonucleic acid. The chromatin material was, therefore, the most efficiently irradiated portion of the cell. The amount of radiation sustained by the nuclei under various experimental conditions is shown in TABLE 2.

The capacity of HeLa cell nuclei to concentrate tritiated thymidine is shown by the concentration factors, which indicate that the amount of radiation

directed to the nucleus is approximately 18 times greater than that contained in an equal volume of media. Regardless of specific activity, the amount fixed during one generation time was approximately 2.8×10^{-9} mg./cell, com-

TABLE 2

IRRADIATION OF CHROMATIN MATERIAL BY TRITIUM AS H^3TDR OR H_2^3O AT 30 HOURS

H^3TDR				H_2^3O		
μc./ml.	Media (rad./day)	Nuclei (rad./day)	Conc. factor	μc./ml.	Media (rad./day)	Nuclei (rad./day)
40	11.5	479	20.8*	90	25.8	25.8
20	5.7	111	19.5	45	12.9	12.9
10	2.9	41	14.4	22.5	6.45	6.45
4	1.2	19	16.4	10.8	3.10	3.10
				5.4	1.55	1.55

* No carrier.

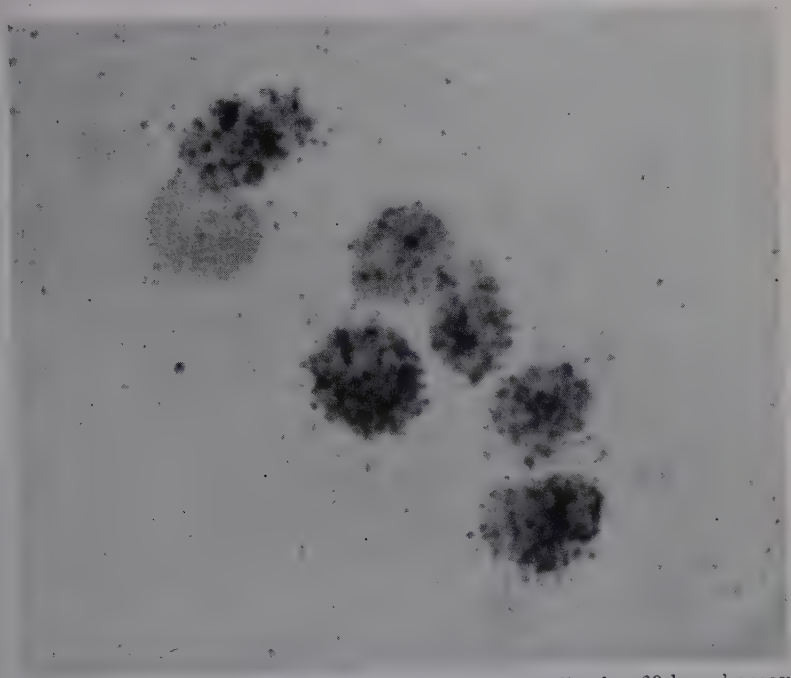


FIGURE 3. Distribution of tritiated thymidine in HeLa cells after 30 hours' exposure.

puted from activity in DNA determined after chemical isolation. These values represent attempts to determine the finite amount of material incorporated by dividing cells and provide a basis for the quantitative interpretation of studies on labeling kinetics.

Of particular interest is the observation that essentially comparable amounts of energy deposited per nucleus by decay of tritiated thymidine and tritium

water exert markedly different biological effects. This implies some mechanism in addition to generalized damage by beta irradiation. An attractive hypothesis is that the *N*-glycoside bond of thymidylate incorporated into the DNA polymer may be disrupted, yielding a "hole" in the template for subsequent replication, a highly specific defect in comparison with the random decay of tritium water.

Autoradiography of the suspended cells under our conditions is not as satisfactory as autography of glass-grown cells. However, the autoradiograph shown in FIGURE 3 is typical of the results seen at 30 hours in which virtually every cell is labeled to some extent. Grain counting due to the geometrical considerations would be of limited significance, and autoradiographs have been employed in these studies only as a qualitative measure of the extent of participation of the population as a whole. The universality of labeled chromatin material in these cells after one generation time indicates that the decrement in growth of the irradiated populations is a mitotic defect, of which the most obvious manifestation is an increase in mean generation time. Thus the studies appear to fit a model of a homogeneous cell population in which DNA synthesis continues but mitosis is inordinately delayed.

These studies demonstrate the applicability of monodispersed cultures of mammalian cells for quantitative studies on radiation effects and suggest the potential of the procedures described for a wide variety of experiments in the fields of radiobiology and fundamental biochemistry.

Acknowledgments

We gratefully acknowledge the assistance of Lora Belle Cole in performing the radioactive determinations and the helpful suggestions of F. N. Hayes during the course of these studies.

References

1. PAINTER, R. B. & R. M. DREW. 1959. Studies on deoxyribonucleic acid metabolism in human cancer cell culture (HeLa). Lab. Invest. **8:** 275–285.
2. PAINTER, R. B., R. M. DREW & W. L. HUGHES. 1958. Inhibition of HeLa growth by intranuclear tritium. Science. **127:** 1244–1245.
3. DREW, R. M. & R. B. PAINTER. 1959. Action of tritiated thymidine on the clonal growth of mammalian cells. Radiation Research. **11:** 535–544.
4. PAINTER, R. B. & J. S. ROBERTSON. 1959. Effect of irradiation and theory of role of mitotic delay on the time course of labeling of HeLa S3 cells with tritiated thymidine. Radiation Research. **11:** 206–217.
5. MCLIMANS, W. F., E. V. DAVIS, F. L. GLOVER & G. W. RAKE. 1957. The submerged culture of mammalian cells: The spinner culture. J. Immunol. **79:** 428–433.
6. VERLY, W. G. & G. HUNEBELLE. 1957. Preparation of H^3 thymidine. Bull. Soc. Chim. Belg. **66:** 640.
7. OTT, D. G., F. N. HAYES & T. T. TRUJILLO. 1959. Liquid scintillation counting of tritium. Biological and Medical Research Group (H-4) of the Health Division—Semiannual Report July through December 1959, Los Alamos Scientific Laboratory Report. **LAMS-2445:** 213–215.
8. DONIACH, I. & S. R. PELC. 1950. Autoradiograph technique. Brit. J. Radiol. **23:** 184–192.
9. SCHMIDT, G. & S. J. THANNHAUSER. 1945. A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in annimal tissue. J. Biol. Chem. **161:** 83–89.
10. SCHNEIDER, W. C. 1945. Phosphorus compounds in animal tissues. I. Extraction and estimations of desoxypentose nucleic acid and pentose nucleic acid. J. Biol. Chem. **161:** 293–303.
11. TOLMACH, L. J. & P. I. MARCUS. 1960. Development of X-ray induced giant HeLa cells. Exptl. Cell Research. **20:** 350–360.

SOME LIMITATIONS OF LABELED COMPOUNDS IN RADIOBIOLOGICAL INVESTIGATIONS OF DEOXYRIBONUCLEIC ACID (DNA) SYNTHESIS

William B. Looney

Radiobiology and Biophysics Laboratory, Department of Radiology, School of Medicine, The University of Virginia, Charlottesville, Va.

Introduction

Labeled compounds have been used extensively in radiobiological investigations to determine the effects of irradiation on biological processes. These investigations have been primarily carried out by biochemical methods. Most biochemical studies using labeled precursors are made by relating the specific activity of the labeled compound in the irradiated biological system to the specific activity of a paired control. The specific activity of the compound in the irradiated biological system related to the specific activity in the control is usually interpreted as a measure of the effects of irradiation on some biological process.

Lajtha[1] has discussed some of the pitfalls of specific radioactivity in measuring synthesis of DNA by labeled precursors of DNA. Specific activity of DNA is dependent upon the fraction of the cells synthesizing DNA, and the amount of DNA synthesized per cell per unit of time. DNA is synthesized during only a small fraction of the total mitotic cycle. Therefore the time of administration and the duration of availability of the labeled precursor of DNA are factors which may also alter the specific activity of DNA in the irradiated and control biological systems.

A combined autoradiographic and cytochemical study of the effects of irradiation on individual hepatocytes of partially hepatectomized rats was made with Barbara Holmes and Colin Campbell at the University of Cambridge, Cambridge, England.[2-4] The quantitative autoradiographic results suggest that a change in the rate of synthesis of DNA occurred in individual hepatocytes during the process of replication.[3] There was a marked increase in the grain counts per nucleus from 15 to 20 hours after hepatectomy. There was a marked decrease in the grain counts per nucleus after 20 hours and before onset of mitosis at 24 hours. The number of hepatocytes labeled during this period was essentially unchanged (FIGURE 1).

The combined autoradiographic and cytochemical studies of DNA synthesis on the same nucleus gave additional evidence suggesting a change in DNA synthetic rate. There was an increasing number of grain counts per nucleus with increasing DNA content up to a point, and this was followed by a decreasing number of grain counts per nucleus with increasing DNA content.[2] These results suggest that the rate of DNA synthesis increases in individual hepatocytes during the first part of the period of replication and decreases during the second part of the period of replication.

Histograms of the log of the grain counts per nucleus in these experiments could be fitted to a normal curve. This was not possible with regard to the histograms of the grain counts per nucleus. Numerous biological data have a

log normal distribution. However, since the quantitative autoradiographic studies indicated that a change in the rate of synthesis occurs during replication, and since the general equation for the normal distribution is exponential, it suggested that the change in synthetic rate of an hepatocyte during replication could be expressed as an exponential function.[2]

It was possible to transform the differential equation for the normal frequency distribution of the log of the grain counts per nucleus into an equation for the change in the rate of DNA synthesis during the process of replication. The DNA content at any time t during the period of replication was predicted by integration of the equation over the interval between the onset of synthesis and time t. The predicted values for the DNA content obtained by integration of the equation for the changing rates of DNA synthesis from the

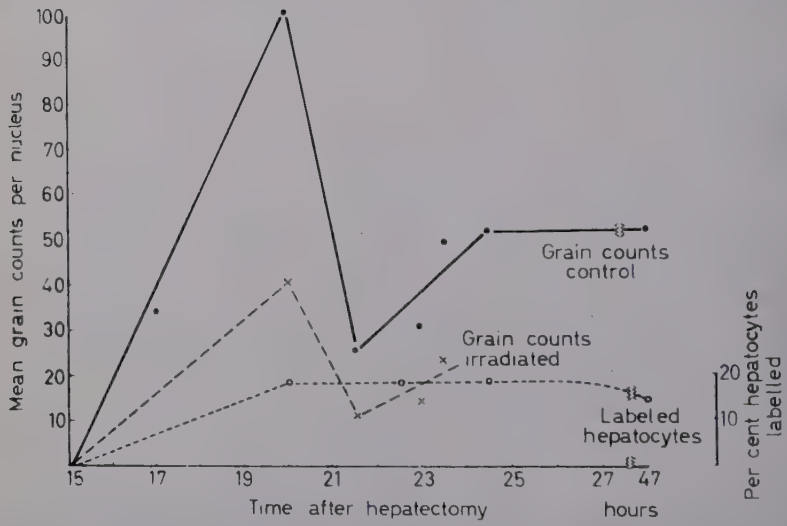


FIGURE 1. Plot of the change in the mean grain counts per nucleus (100 nuclei) and percentage of hepatocytes labeled after hepatectomy. Reproduced from Looney[3] by permission of Butterworth and Co., London, England.

autoradiographic results were expressed in relative units between 0 and 1. The experimentally determined values from the combined autoradiographic and cytochemical studies were expressed in relative values between 4N and 8N as being between 0 and 1. These experimentally determined mean values were obtained by giving tritiated thymidine and sacrificing the animal at different intervals during the period of replication.

The experimental results from the cytochemical studies have been fitted to a curve from an integrated equation for changing rates of DNA synthesis. Thus the theoretical curve predicted from the autoradiographic results agrees with the experimentally determined cytochemical results. The mean time for replication of DNA content of an hepatocyte was found to be 8 hours.

This combined autoradiographic and cytochemical study was used also in the study of the effects of irradiation following 3000 r of X radiation. Labeling of the hepatocytes with tritiated thymidine provides a means for identify-

ing the hepatocytes synthesizing DNA at the time of irradiation. Sacrificing the rats at varying times after irradiation and thymidine administration, and measuring the DNA content of the labeled nuclei microspectrophotometrically permits the determination of the changing rate of the DNA content of the hepatocytes following irradiation.

The mean time for replication of the DNA content of an hepatocyte of 8 hours in the paired controls was increased to 13 hours in the irradiated animals (FIGURE 2). The shape of the exponential curve for the changing DNA content during the period of replication following irradiation was similar to the shape of the curve in the controls.

The fact that the mean time for the completion of DNA synthesis increased from 8 to 13 hours after 3000 r of irradiation demonstrates how the use of labeled precursors of DNA to measure the irradiation effects on DNA synthesis can give erroneous results by using biochemical methods only. If the labeled precursors are injected into the irradiated and paired control animals

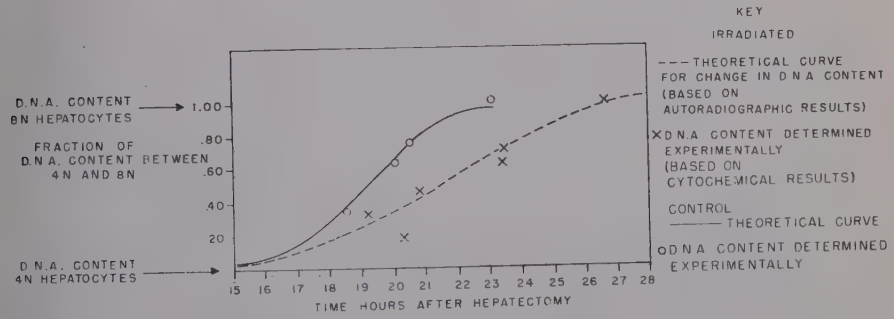


FIGURE 2. Change in DNA content with time after hepatectomy measured microspectrophotometrically in tritiated thymidine labeled hepatocytes. Reproduced from Looney[2,4] by permission of the *Proceedings of the National Academy of Sciences.*

between 8 and 13 hours after irradiation, the labeled precursors will be incorporated into a second cycle of hepatocytes synthesizing DNA in the paired control. In addition, following the completion of synthesis of DNA by the first cycle of hepatocytes, the nonparenchymal cells as well as a second cycle of hepatocytes begin synthesis. The evaluation of effects of irradiation by labeled precursors with biochemical methods alone would obviously give erroneous results. The labeled precursors would be incorporated into different cell types, as well as a second cycle of hepatocytes in the paired control. The labeled precursors in the irradiated animal would still be incorporated into the first cycle of hepatocytes.

This combined autoradiographic and cytochemical study has demonstrated that the combined study is superior to either the autoradiographic or the cytochemical study made independently on irradiation effect within populations of cells. The results of the cytochemical studies alone would suggest that irradiation had inhibited DNA synthesis in some of the nuclei. However, the autoradiographic studies have shown that this interpretation is incorrect since no significant difference has been shown between the percentage of the hepatocytes synthesizing DNA in the irradiated and control animals. The autoradio-

graphic studies have shown that the rate of synthesis of DNA is reduced in all nuclei (FIGURE 3). This apparent inhibition is the result of reduced DNA synthesis to such a degree that the change cannot be detected by the microspectrophotometric measurements.

Time and Rate of DNA Synthesis in Irradiated and Paired Control Populations of Cells

Results during DNA synthesis. It is implicitly assumed in the interpretation of the results of most investigations that the rate change in the DNA con-

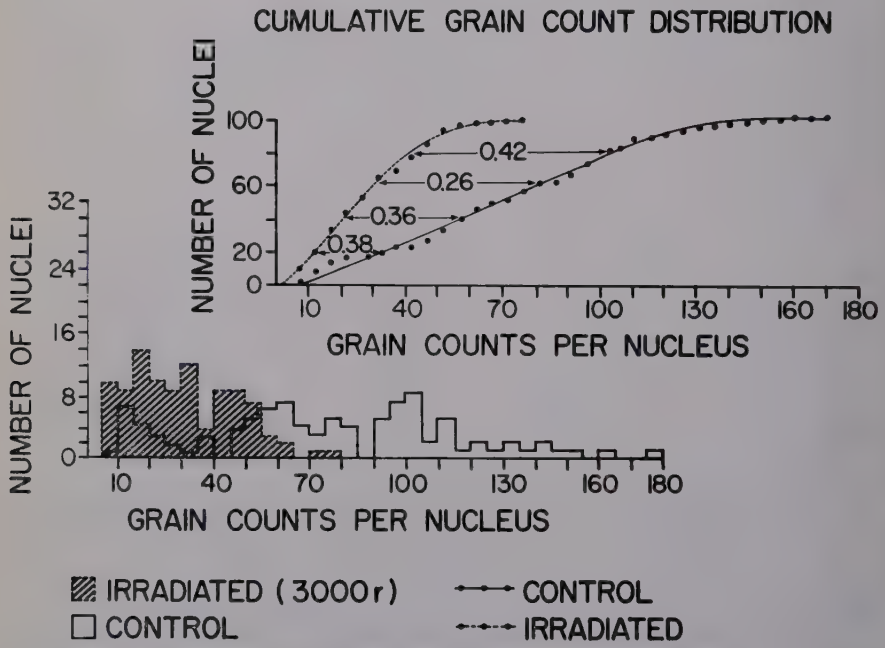


FIGURE 3. Histogram of grain count distribution in hepatic nuclei in a control and irradiated rat after tritiated thymidine administration. Reproduced from Looney,[12] by permission of Georg Thieme Verlag.

tent of cells is constant during the process of replication. The results of quantitative autoradiographic, and combined autoradiographic and cytochemical studies of DNA replication in partially hepatectomized rats suggest that change in the DNA content of rat hepatocytes is exponential rather than linear.

It is apparent that differences in the time sequence of irradiated and control biological systems would give differences in the ratios of the labeled compounds in the irradiated and control biological systems. Differences in the rate of uptake in irradiated and control biological systems is diagrammatically illustrated in FIGURE 4. It is assumed that irradiation has reduced the rate of DNA synthesis by one half.

If the rate of DNA synthesis was increasing in the paired control population

of cells, and the rate of DNA synthesis in the irradiated cells was decreasing, the relationship of the rate of DNA synthesis of the irradiated to the paired control would be different than if the reverse were true. If the labeled precursors in the paired control were administrated and left for 1 hour the rate would increase from 2 to 4. The rate of DNA synthesis in the irradiated cells would be reduced during this 1 hour period from 2 to 1. Thus the rate in the

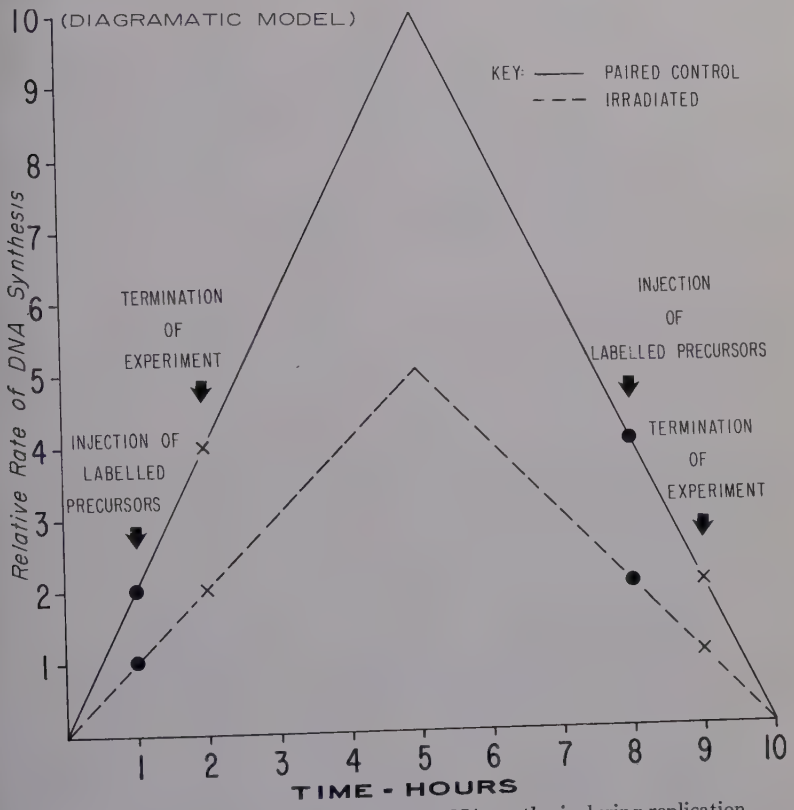


FIGURE 4. Relative changes in rate of DNA synthesis during replication.

paired control would be 4 times the rate in the irradiated system at the termination of the experiment.

If the rate of DNA synthesis was decreasing in the paired control population of cells, and the rate increasing in the irradiated population of cells, then the rate in the paired control would decrease from 4 to 2 and the rate in the irradiated cells would increase from 1 to 2. Thus the rate in the paired control would be the same as the rate in the irradiated population of cells. Therefore the results from the use of labeled precursors would suggest that irradiation caused no change in the rate of DNA synthesis, when in actuality the irradiation had reduced the rate of synthesis one half.

Results after mitotic division. Quantitative autoradiographic studies have

also shown that following mitosis further misinterpretation of the results may be made. If the cells in the paired control divide prior to termination of the experiment and the irradiated cells do not, it would suggest that irradiation has caused a 50 per cent increase in DNA synthesis (this is assuming that irradiation caused no effect on DNA synthesis). The correct interpretation would be that a 50 per cent decrease in tritiated thymidine uptake had occurred in the divided cells of the control.

It becomes evident that after the second division of the nuclei in the control animals, without division in the irradiated animals, a 50 per cent reduction in the specific activity of the controls would occur. After the second division of the labeled hepatocytes of the control, the biochemical results could also be misinterpreted if it were assumed that irradiation had caused a 50 per cent increase in DNA synthesis rather than the correct inference that a 50 per cent decrease in the tritiated thymidine uptake had occurred in the divided hepatocytes in the control (again assuming that irradiation caused no effect on DNA synthesis).

Fifty Per Cent Reduction of DNA Precursors with Different Irradiation Doses

Results of several investigations have appeared in the literature which report that relatively constant reduction in DNA synthesis of approximately 50 per cent occurs with large differences in radiation dose. The analysis of the cumulative distribution of the grain counts per nucleus showed a relatively constant ratio between the irradiated and control curves at the same ordinate. These results suggest that the reduction in the rate of DNA synthesis immediately following irradiation is proportionate to the rate of synthesis at the time of irradiation (see FIGURE 3). The fact that relative rates of DNA synthesis in different hepatocytes varied by a factor of 10 or more suggests that no qualitative difference exists in the effect of irradiation on the various stages of DNA synthesis. This uniform shift in the frequency distribution curve was also found in quantitative autoradiographic studies with tritiated thymidine following the irradiation of rapidly proliferating tissues in mice and rats. The effect of irradiation on DNA synthesis was well established with doses as little as 100 r. The effects were demonstrated less than one hour after irradiation.[5]

The results of combined autoradiographic and cytochemical studies in regenerating liver have shown that misleading interpretations may be made if either the precursors are left for a longer period of time after irradiation or the labeled precursors are injected at long periods of time following irradiation. The previously reported results have been reviewed in an attempt to determine if the "50 per cent effect" is one of misinterpretation or a real qualitative difference in the response of DNA synthesis to irradiation after it has been reduced one half.

It was found in one report that a 50 per cent reduction in uptake of P-32 nto the DNA of a tumor occurred after 4500 r. No further decrease in uptake occurred after 9000 r. These results were based on the mean uptake of P-32 at various time intervals between 0 and 24 hours. In view of these more recent experiments it is considered that valid conclusions cannot be drawn from these results.[6] Comparison of uptake of P-32 in different populations of cells in the irradiated and control tumors was in all probability being made.

Differences in dose-response curves have been found, and these results have been interpreted as showing the effects of irradiation on different processes involved in the process of DNA replication. Ord and Stocken[7] have shown that the dose-response curve for reduction in DNA synthesis following irradiation can be resolved into two distinct components from biochemical studies. They suggest that the rapid decrease on the initial part of the dose-response curve might be attributed to inhibition of nuclear phosphorylation and the slower component to progressive damage of the template. Clifton and Vermund (personal communication) have shown that a linear relationship exists between the suppression of the incorporation of P-32 into mouse mammary tumors and the log of the dose. Examination of the results of Beltz *et al.*,[8] shows that a dose-response curve was obtained which was in some respects similar to the dose-response curve found by Ord and Stocken. Reduction in DNA synthesis was proportional to the irradiation dose over the range from 375 r to 3000 r. The recent studies of the effects of irradiation on DNA synthesis with labeled precursors demonstrates, therefore, that a 50 per cent effect is unlikely. The fact that a relatively constant 50 per cent depression of uptake of DNA was found in earlier experiments after marked differences in radiation dose was in all probability the result of the experimental design and the differences in radiation effects on different processes involved in DNA synthesis.

Uptake of Labeled Precursors of DNA in in Vitro Systems

Crathorn and Shooter[9] have examined tritiated thymidine incorporation in Ehrlich and Landschutz ascites cells *in vitro* to study the relation between the assimilation of labeled precursors and their incorporation into DNA. They found that thymidine is rapidly taken up by the cells but the rate of incorporation into DNA is much slower. It was also found that after one hour the cells failed to take up more thymidine or synthesize DNA in a media of 50 per cent ascitic fluid and 50 per cent fortified Hanks' media. They suggest that either some change occurs or some metabolic process is lacking in the media which prevents the cells from either accumulating thymidine or synthesizing DNA.

Nuclei were separated by the method of Allfrey *et al.*[10] and almost no activity was found in the nonnuclear fraction. The autoradiographs also demonstrated that the activity was confined to the nucleus. The activity was not diminished either by standing or by the addition of inactive thymidine.

Treatment of the nuclei with ultrasonics and centrifugation caused a considerable loss of nuclear activity. It was found that three fourths of the activity could be accounted for either as thymidine, thymidine mono-, di-, or triphosphate. The other one fourth of the thymidine was incorporated into the DNA molecules after 15 min. of incubation. After 60 min. of incubation approximately two thirds of the activity was found in the isolated DNA and one third in the precursors. Since the total activity in the cells was approximately the same for 15- and 60-min. incubation periods, and since the same per cent of cells were found by autoradiography to be labeled, the authors suggest that the labeled thymidine is transferred to the nucleus and an equilibrium rapidly established between thymidine, thymidylic acid, and the di- and triphosphate derivatives.

Alpen *et al.*[11] have recently reported the results of their study on the effects of irradiation on DNA synthesis in rat lymphoid tissue. They were able to show that rather significant differences existed between the response measured autoradiographically and the response as measured by specific activity using P-32. They consider that the tritiated thymidine findings more nearly represent the true state of DNA synthesis because of the inherent limitations of the biochemically determined DNA–P-32 specific activity.

They found a depression of DNA synthesis shortly after irradiation was followed by recovery. This was followed by a second depression of synthesis 4 to 5 days after irradiation. They suggest that the apparent recovery may be abortive and that latent damage to the cell may result from the second depression of synthesis.

There was a marked difference in the *in vivo* and *in vitro* response of lymphocytes to irradiation. Only 300 r total body radiation caused a 50 per cent depression of DNA synthesis in lymphocytes, while 2000 r produced only a 27 per cent depression of DNA synthesis of the lymphocytes *in vitro*. Alpen *et al.* suggest that when lymphocytes are studied *in vitro* a very large radiosensitive component of the metabolic processes is lost.

They consider that the radiosensitive component that is lost is comparable to the first component of the dose-response curve of Ord and Stocken. The more radioresistant component found in the *in vitro* system is considered to be comparable to the second component of this dose-response curve.

Summary and Conclusions

The results of recent biophysical and biochemical investigations have demonstrated that careful experimental design is necessary to avoid erroneous or misleading results. The ratio of the specific activity of the labeled compound in the irradiated biological system to the labeled compound in the control is usually interpreted as a measure of the effects of irradiation on a biological process. Since specific activity of DNA is dependent upon the fraction of cells synthesizing DNA and the amount of DNA synthesized per cell per unit of time, information is needed about DNA synthesis within populations of cells. Biochemical studies alone do not provide this information.

The rate of synthesis during DNA replication may be exponential. Differences in the time sequence between the irradiated and control populations of cells of synchronous biological systems will give differences in the ratio of the specific activities of labeled compounds.

A review of the results reported on the effects of irradiation on DNA synthesis as measured by labeled precursors suggests the following. The finding of two components of the dose-response curve measured with labeled compounds indicates that irradiation affects more than one process involved in the biosynthesis of DNA. Labeled precursors injected during or shortly after irradiation should give results which should more accurately reflect the effects of irradiation on the DNA synthetic process. Relative changes in the rate of synthesis and relative changes in the population of cells in the irradiated and paired control biological systems make quantitative results between the dose delivered and the effects of DNA synthesis unlikely if the labeled precursors are not administered immediately after irradiation. Labeled precursors in-

jected from several hours up to days after irradiation make the results of the effects of irradiation on DNA synthesis difficult or impossible to evaluate. The finding that DNA synthesis is depressed for days after irradiation probably involves the effects of irradiation on cell processes other than DNA synthesis. Depression of the uptake of the labeled precursors is in all probability an indirect effect mediated through disturbances in other cell processes such as mitosis.

It is therefore considered that coordinated biophysical studies on individual cells and biochemical studies on total populations of cells offer one of the best approaches to obtain a better understanding of irradiation effects on DNA. Coordinated *in vivo* and *in vitro* studies should be more effective than either study made independently in the attempt to gain a better understanding of the biological effects of irradiation.

References

1. Lajtha, L. G. 1958. Pitfalls of specific radioactivity in measuring synthesis of deoxyribonucleic acid. Nature. **181:** 1609.
2. Looney, W. B. 1960. The replication of deoxyribonucleic acid in hepatocytes. Proc. Nat. Acad. Sci. **46:** 690–698.
3. Looney, W. B. 1960. A cytochemical and autoradiographic study of deoxyribonucleic acid synthesis in individual nuclei and the effects of irradiation on synthesis. *In* The Cell Nucleus. **:** 98–125. J. S. Mitchell, Ed. Butterworth. London, England.
4. Looney, W. B., R. C. Campbell & B. E. Holmes. 1960. The effect of irradiation on the replication of deoxyribonucleic acid in hepatocytes. Proc. Nat. Acad. Sci. **46:** 698–708.
5. Quastler, H. 1961. X-irradiation and DNA synthesis. *In* Abstracts Fifth Annual Meeting of the Biophysical Society. **:** SD-5. St. Louis, Mo.
6. Vermund, H., C. P. Barnum, R. A. Huseby & K. W. Stenstrom. 1953. The effect of roentgen radiation on the incorporation of radiophosphorus into nucleic acids and other constituents of mouse mammary carcinoma. Cancer Research. **13:** 633–638.
7. Ord, M. & L. A. Stocken. 1958. Studies in synthesis of deoxyribonucleic acid. Nature. **182:** 1787–1788.
8. Beltz, R. E., J. Van Lancker & V. R. Potter. 1957. Nucleic acid metabolism in regenerating liver. IV. Effect of X-radiation on the whole body on nucleic acid synthesis *in vivo*. Cancer Research. **17:** 688–697.
9. Crathorn, A. R. & K. V. Shooter. 1960. Uptake of thymidine and synthesis of deoxyribonucleic acid in mouse ascites cells. Nature. 187: 614–615.
10. Allfrey, V. G., A. E. Mirsky & S. Osawa. 1957. Protein synthesis in isolated nuclei. J. Gen. Physiol. **40:** 451–490.
11. Alpen, E. L., E. H. Cooper & H. Barkley. 1960. Effects of ionizing radiation on rat lymphoid tissue *in vivo*. Int. J. Rad. Biol. **2:** 425–439.
12. Looney, W. B. 1959. Proc. IXth International Radiological Congress. Georg Thieme Verlag. Stuttgart, W. Germany. In press.

MAMMALIAN CELL-CULTURE METHODS IN INVESTIGATING MODIFICATION OF THE RADIATION RESPONSE*

Robert Bases
Sloan-Kettering Institute for Cancer Research, New York, N.Y.

Introduction

The reproductive capacity of single mammalian cells to produce macroscopic colonies *in vitro* was immediately exploited by Puck and Marcus to study the action of ionizing radiation on cells.[12-14] Other parameters besides reproductive ability have been studied. However, disturbances in synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein by mammalian cells appeared much less sensitive to radiation.[19] Indeed, these constituents may vary only secondarily.[17] Radiation sterilized cells may divide abortively several times and produce considerable amounts of cell constituents.[5,13,14] Therefore, reliance on variation in constituents or cell population as parameters of effect may lead to serious underestimates of sterilization.

The benefit that could result from improved techniques in clinical radiation therapy of human malignancy generates much interest in radiation sterilization. The purpose of this report is to describe our own attempts at modifying radiation sterilization of cells *in vitro*. Results of others who used *in vivo* and *in vitro* techniques will be compared. No mention will be made of mutation, another class of radiation response. Cell-culture methods are becoming available, but the application is almost entirely unexplored.

Materials and Methods

Experiments were performed with the HeLa cells of the S-3 subline grown in Eagle's medium[7] with 10 per cent undialized human serum. The designation S-3-1, S-3-2 has no connotation outside this work and is used only to discriminate between our 2 lines.

Generation time was 22 hours ± 1 hour in an incubator adapted to provide a moist atmosphere of 5 per cent CO_2, 95 per cent air. Cells were plated in 100-mm. Petri dishes or 4 oz. prescription bottles. Cells were removed from their bottles and from each other with Versene. A more complete description of methods has been given by Bases.[4,5]

Viability: the capacity of single cells to produce macroscopic clones upon 12 days of incubation. Puck's criteria were followed in scoring.[13] Standard error of the mean number of clones in replicate dishes was usually about 5 per cent.

Plating efficiency: seventy to 100 per cent of control single cells were capable of clone formation. Irradiations were performed with a Van de Graaf accelerator operating at 2.5 Mev, 730 μamps, target distance 100 cm. delivering 432 rpm. S-3-1 plated with 70 to 100 per cent efficiency until an accidental period of inadequate feeding when it changed to 30 to 70 per cent. S-3-2 had the original 70 to 100 per cent plating efficiency but varied in radiation sensi-

* The experimental work described in this paper was performed at the National Cancer Institute, Public Health Service, Bethesda, Md.

tivity as described in the text. Actinomycin D is an antibiotic from *Streptomyces parvullus*.

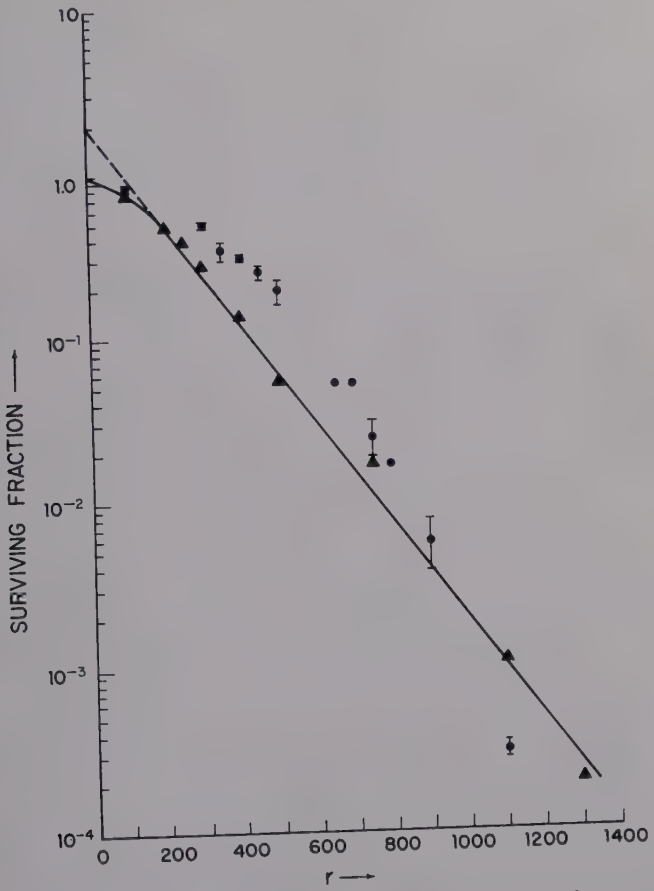


FIGURE 1. Dose response curves. Key: ▲ = S-3-1, each symbol represents 2 to 10 experiments; ● = S-3-2, each symbol represents survival data from 2 to 18 experiments. Standard errors of the mean are indicated for doses having data from 3 or more experiments; 100 r, 300 r, 900 r, 1100 r values represent 12, 18, 8, and 4 experiments respectively. Determinations in individual experiments were performed in triplicate.

Results and Discussion

FIGURE 1 shows a dose-response curve for S-3-1. Each symbol represents 2 to 10 experiments accumulated over an 18-mo. period. The curve has an initial shoulder but then is linear. The mean lethal dose (MLD) is that needed to reduce the surviving fraction to 37 per cent or 1/e on the linear portion of the curve; it was 150 r. The curve extrapolates to 2. On the same graph are data from 2 to 18 experiments with S-3-2 from an 8-mo. period. These data did not fall on a straight line but in every individual S-3-2 or S-3-1 experiment a shoulder and a linear-log portion were obtained.

Early S-3-2 experiments collectively gave linear-log curves as in that of FIGURE 1. However, S-3-2's MLD began to vary between 100 r and 150 r and its extrapolation number between 4 and 10, but the S-3-2's degree of variability in MLD was never seen with S-3-1. The cause of it is unknown. These variabilities among experiments with S-3-2 and the uniformity among S-3-1 are worthy of note because both cell lines were from the same source and the differences in radiation sensitivity appear to have arisen spontaneously. The difference in MLD between S-3-1 and S-3-2 were of the order of difference believed to have been selected for by irradiation in the experiments of others.[18] Our own attempts to select for radiation resistant cells were unsuccessful.[5]

TABLE 1*
ACTION OF IONIZING RADIATION ON MAMMALIAN CELLS

Type	MLD†	Extrapolation No.	References
In vivo:			
Mouse marrow	115 rad	2	Till and McCulloch[16]
CBA leukemia	165 r‡	2	Hewitt and Wilson[10]
P. 388 leukemia	160 rad	1 to 2	Andrews and Berry[1]
In vitro:			
Human, various	50 to 166 r	1 to 2	Puck *et al.*[14]
HeLa S-3	138 rad	2	Puck and Marcus:[13] Morkovin and Feldman[12]
HeLa S-3	~100 r	~6	Bagshaw[2]
HeLa S-3-1	150 r	2	Bases[4]
HeLa S-3-2	100 to 150 r	4 to 10	Bases[4]
Kidney	135 rad	—	Barendsen *et al.*[3]
Kidney	(65 rad, 3.4 mev α)	—	Barendsen *et al.*[3]
Liver	119 rad	2	Dewey[6]
Marrow D98	180 r	—	Erickson and Szybalski[9]
Mouse L fibroblast	240 rad	2	Whitmore *et al.*[17]
Hamster, lung, ovary	100 to 150 rad	5 to 10	Elkind and Sutton[8]
Chick (fibroblast) *in vitro*	600 r	—	Rubin and Temin[15]

* Data from various investigators who use MLD and extrapolation numbers to define radiation sensitivity.
† Mean lethal dose = dose leaving 1/e or 37 per cent survivors.
‡ Exposure dose, X rays.

TABLE 1 shows data from various investigators who use MLD and extrapolation numbers to define radiation sensitivity. There is remarkable agreement among results of *in vitro* work using methods similar to those described above. The groups who designed *in vivo* experiments to provide data about MLDs and extrapolation numbers obtained results identical with that from *in vitro* experiments.[1,10,16] This is both remarkable and gratifying indeed.

A possible limitation of all these studies is the following. The cells were irradiated and assayed while dividing at a maximum rate. This was clearly true for *in vitro* work. Only Elkind and Sutton's work[8] gave a clue to the radiation sensitivity of lag-phase cells. These investigators found that the MLD decreased slightly when lag-phase cells entered log phase. Hewitt and Wilson[10] assayed irradiated mouse leukemia cells growing at what must have been a maximum rate in host mice that died in 3 or 4 weeks. Till and

McCulloch[16] assayed the effect of radiation on the ability of single normal mouse-marrow cells to repopulate areas of spleens of supralethally irradiated mice. Macroscopic colonies of viable cells appeared in 10 days. These cells must have been dividing at a maximum since the colonies appear to have arisen from single cells.

Despite the restriction we have some good justification for using *in vitro* studies as guides to *in vivo* radiation. Thus techniques are available for quanti-

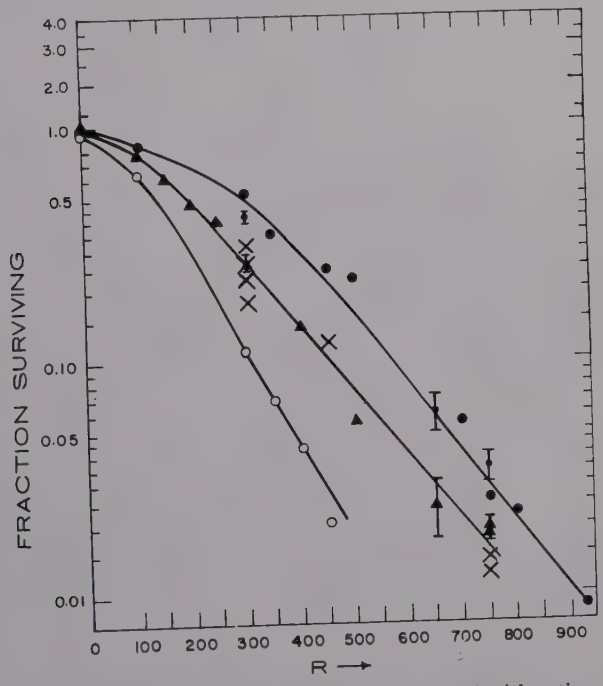


FIGURE 2. Dose-response of HeLa cells treated or untreated with actinomycin D. Key ● = S-3-2, mean of 2 to 7 experiments represented by each symbol; ▲ = S-3-1, mean of 2 to 7 experiments represented by each symbol; ○ = S-3-2, survivors of 1 μg./ml. actinomycin D, the mean of 4 experiments represented by each symbol; × = S-3-1, fraction surviving irradiation of large populations of cells in logarithmic growth phase. Each symbol represents a separate experiment. The contents of irradiated bottles were assayed for clone-forming cells after irradiation. Similar results were obtained with S-3-2. Standard-error symbols are for a single experiment in which curves for S-3-2 and S-3-1 were compared. Reproduced by permission of *Cancer Research*.

tative study of modification. Altering MLD or extrapolation number by various means produces significant variations in the per cent of a treated population that survives a given dose.

FIGURE 2 demonstrates this. Two curves should be compared. The lowest is a composite curve obtained from 4 separate dose-response curves for S-3-2 cells surviving 1 μg./ml. of actinomycin D for 75 min. Cells were suspended and irradiated after drug was removed. The uppermost is from control S-3-2 dose-response curves of these 4 experiments and data from 3 other S-3-2 curves. Administering 500 r to drug survivors sterilized as large a proportion of the

population as did 800 r to the control cells. The drug effect was more evident at higher radiation doses. There was a remarkable decrease in the shoulder and a decrease in extrapolation number, while the MLD appeared to have decreased only a little. Results of the individual experiments were similar.

In addition to demonstrating enhanced radiation sensitivity FIGURE 2 illustrates 2 other facts. In a single experiment indicated by standard-error

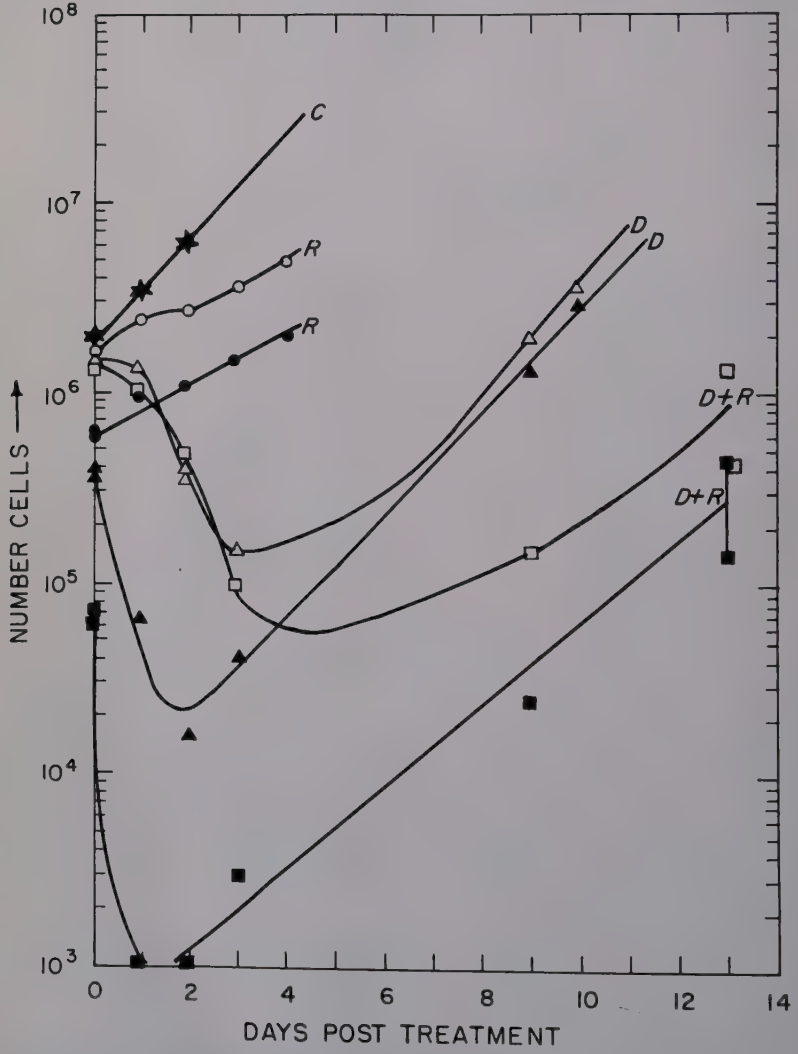


FIGURE 3. Effect of actinomycin D and irradiation on cells. Key: open symbols represent total cell counts; solid symbols represent the number of viable ones (clone forming cells); C = controls (duplicate values too close to demonstrate; plating efficiency, 109, 104, 91 per cent on days 0, 1, 2); R = irradiated, 400 r, 2.5 mev; D = actinomycin D, 1 μg./ml. for 75 min.; and D + R = D followed by R. Media were changed in all flasks remaining on days 1, 2, 3, 9, 10, and 11. Reproduced by permission of *Cancer Research*.

symbols S-3-2 is shown slightly less sensitive to radiation than S-3-1. This appears due to a higher extrapolation number.

Finally, there are data shown obtained by assaying large populations (that is, 1 million S-3-1 cells) for survivors after irradiation in log phase. The fractions surviving in 7 such experiments coincided with the curve for irradiated *single* S-3-1 cells. This shows that radiation sensitivity is independent of the number of cells in a population that is irradiated.

FIGURE 3 shows growth curves made by counting the total cells and assaying the viable ones from replicate bottles at intervals after treatment with X ray or actinomycin D, 1 μg./ml., for 75 min., or both. It is another way of showing enhanced radiation effect.

Only a small fraction of drug-treated or irradiated cells was viable. This fraction eventually repopulated the bottles. The surviving fraction might be increasing even when the total cell population was declining or unchanged. An irradiated population may at first increase nearly as fast as the controls even when most cells are sterile. FIGURE 3 emphasizes that determination of colony-forming ability is a much more exact criterion of effect than a mere count of the total population.

Viability curves for drug-treated populations declined for the first two days after actinomycin was removed. This shows that despite rinses and media changes, residual drug remained in or on these cells and continued to sterilize cells that would have been scored as viable if assayed earlier.

This finding in no way invalidates conclusions based on the data of FIGURE 2 because no assumptions were made regarding presence or absence of residual actinomycin D when diluting drug-treated populations.

The continuing action of actinomycin D as demonstrated in FIGURE 3 shows it is not immediately lethal on contact and this suggests, but does not prove, that its mechanism of action is like that of an antimetabolite rather than an alkylating agent.

Survivors of irradiation were much more sensitive to actinomycin D than their controls: this is shown in FIGURE 3 in two ways. During the first two days after treatment the survivors of irradiation were sterilized four times as fast by actinomycin D as were the controls. Curve D + R for viable cells, solid squares, dropped four times as steeply as did curve D, solid triangles.

The second way of demonstrating enhanced sensitivity from FIGURE 3 is shown in TABLE 2, data of day 0. This compares the fraction of a population surviving double treatments with the product obtained by multiplying by each other the fractions surviving each treatment. The observed surviving fraction was about one-half that expected. The fraction of a cell population that survives an initial treatment is the new population at risk for a succeeding treatment, since a cell may be sterilized only once. Therefore one may properly calculate the surviving fraction as the product rather than the sum of these fractions. Applications of this method to a study of 6-mercaptopurine and radiation did not show enhancement.[5]

Sterilization of cells by progressive exposure to actinomycin D as shown in FIGURE 3 was a hint that the drug's major action was not because of the 1 μg./ml.

for 75 min. but due to a much smaller amount of residual drug in contact with the cells over 12 days of incubation in the assay plates. FIGURE 4 corroborates this. Therefore the experiment of FIGURE 5 was carried out.

Radiation dose-response curves were determined for cells inoculated in

TABLE 2
TREATMENT SURVIVORS AS A FRACTION OF CONTROLS

Treatment	Surviving fraction	
None (controls)	1.17	0.94
400 r	0.34	0.35
Actinomycin D	0.20	0.22
Both, expected	0.068	0.077
Both, found	0.036	0.040

Data from day 0 of the experiment shown in FIGURE 3. See the text for details. Duplicate cell cultures received the treatments shown. Each culture was assayed for survivors in triplicate, and the mean value for each is shown.

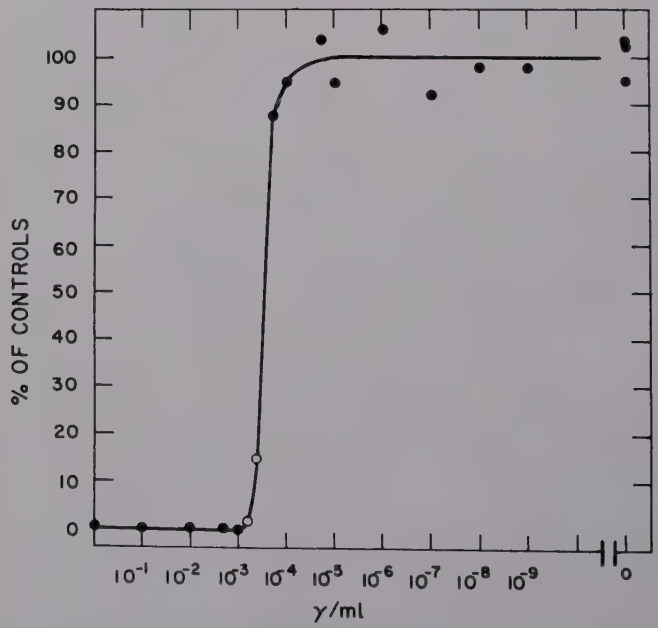


FIGURE 4. Clone formation of single HeLa cells (S-3-2) in varying concentrations of actinomycin D. Key: ●, one experiment; and ○, another experiment. Reproduced by permission of *Cancer Research.*

dishes containing 4×10^{-4} μg./ml. of drug. Only 15 per cent of the cells survived the drug and the curve is for the radiation survival of this fraction. The survival values were the same if drug was added before or after irradiation. The common feature is that actinomycin D was present throughout the 12-day incubation period. Enhanced sensitivity is evident in both cases.

This corroborates the impression previously gained from FIGURE 3 that

radiation survivors are abnormally sensitive to the drug. Apparently actinomycin D acts on the postirradiation state of cells. Perhaps actinomycin D suppresses repair. Normal sensitivity to actinomycin D returns within a few generation times after irradiation. Bagshaw[2] working with similar techniques has shown that a similar potentiating effect is obtained with 5-fluorouracil (5-FU) at doses that are not themselves toxic.

Ionizing radiation itself appears to render survivors no more or no less sensi-

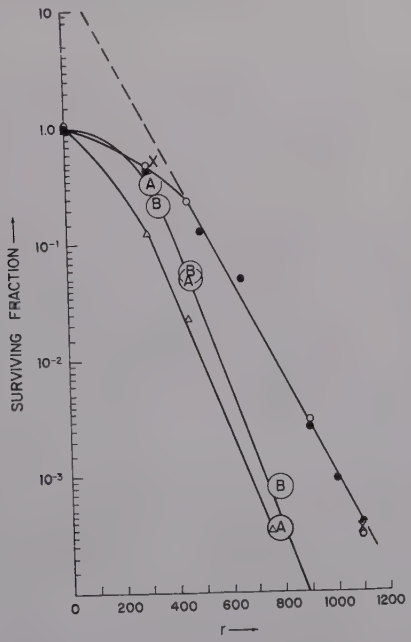


FIGURE 5. Dose response curves. Key: △ = D_2O treated S-3-2; irradiation was in normal medium; this is the curve for the 21 per cent that survived 14 hours in 85 per cent D_2O medium; ○ = control S-3-2; plating efficiency, 103 per cent; this is the control for the above D_2O treated S-3-2; ● = another S-3-2 experiment with the same batch of normal medium as in the above experiment; plating efficiency, 100 per cent; Ⓐ = S-3-2, 4×10^{-4} μg./ml. actinomycin D added to dishes 10 min. after irradiation; Ⓑ = S-3-2, 4×10^{-4} μg./ml. actinomycin D added to dishes 10 min. before irradiation; and × = control S-3-2 for the before/after experiment. Reproduced by permission of *Cancer Research*.

tive to subsequent irradiation,[5] provided a sufficient time elapses for postirradiation repair to complete itself.[8]

D_2O: Exposure to deuterium oxide, D_2O, irreversibly sterilizes cells. FIGURE 6 illustrates this, and FIGURE 5, showing a radiation dose-survival curve for D_2O survivors, illustrates a good example of potentiation due to presensitization. A well defined case of presensitization is that provided by Erikson and Szybalski,[9] who showed that incorporation of 5-halogenated thymidine analogs (5-chloro, 5-bromo or 5-iododeoxyuridine) into DNA of mammalian cells increases their sensitivity to X ray (MLD changed from 180 r to 75 r for IUDR). Szybalski *et al.*[9] have also shown that 10 per cent glycerol in the medium decreases sensitivity of cells that have or have not incorporated the analogues.

The MLD is increased to about double that without glycerol. Another protective agent is aminoethylisothiouronium bromide (AET).[5]

Dewey[6] working with human liver cells *in vitro* showed that irradiation in

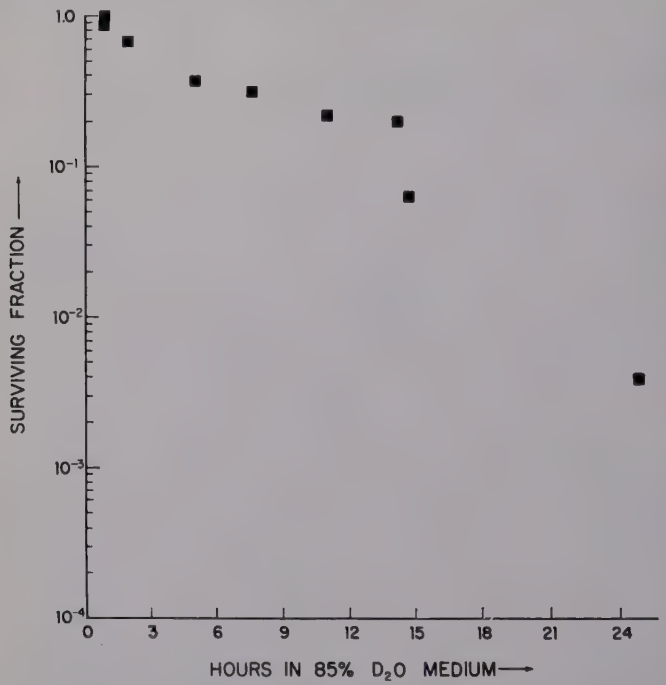


FIGURE 6. Irreversible sterilization by exposure to Eagle's medium with 85 per cent of water replaced by D_2O S-3-2.

TABLE 3
MODIFYING RADIATION STERILIZATION OF CELLS

	Before radiation	During radiation	After radiation
Enhancing	XUDR* D_2O (Erikson *et al.*;[9] Bases[4])	O_2, NO_2, D_2O (Dewey;[6] Bases[4])	Actinomycin D 5-FU (Bases;[4] Bagshaw[2])
Suppressing	AET (Bases[5])	N_2 Glycerol 10 per cent (Dewey;[6] Erikson and Szybalski[9])	"Natural repair" (Elkind and Sutton[8])

* 5-Halogenated derivative of deoxyuridine (x = Cl, Br, I).

NO_2 or O_2 decreased the MLD to $\frac{1}{2.2}$ times that obtained in N_2. This agrees with a 2.3-fold increase in MLD obtained by Hewitt who irradiated leukemic cells in anoxic dead mice rather than in live, well oxygenated mice.[11]

TABLE 3 is only intended to catalogue present knowledge of the sequences of some radiation-modifying agents. It is not meant to suggest the mechanisms involved.

Two areas that deserve further study in the future are (1) relative sensitivity of cells in various stages of the mitotic cycle, and (2) factors determining the radiation repair process.

A great problem hovers around the application of these agents to clinical situations. This is the old one of differential toxicity. Can we protect the normal tissues while simultaneously sensitizing the malignant ones? That is a problem that will be defined by the agents involved, and may not be as easily solved as that of modifying the radiation response in systems examined thus far.

Summary

Single-cell techniques are of great value in studying radiation sterilization. Examples of the use of these techniques in the study of modification of the radiation response are given.

References

1. Andrews, J. R. & R. J. Berry. 1961. Quantitative studies of radiation effects on cell reproductive capacity in a mammalian transplantable tumor system *in vivo*. Ann. N.Y. Acad. Sci. **95**(2): 1001–1008.
2. Bagshaw, M. 1961. Possible role of potentiators in radiation therapy. Am. J. Roentgenol. Radiation Therapy and Nuclear Med. **85:** 822–833.
3. Barendsen, G. W., T. L. J. Beusker, A. J. Vergroesen & L. Budke. 1960. Effects of ionizing radiation on human cells in tissue culture. Radiation Research. **13:** 841–849.
4. Bases, R. E. 1959. Modification of the radiation response determined by single cell techniques: actinomycin D. Cancer Research. **19:** 1223–29.
5. Bases, R. E. 1959. Some applications of tissue culture methods to radiation research. Cancer Research. **19:** 311–15.
6. Dewey, D. L. 1960. Effect of oxygen and nitric oxide on the radiosensitivity of human cells in tissue culture. Nature. **186:** 780–782.
7. Eagle, H. 1955. Nutrition needs of mammalian cells in tissue culture. Science. **122:** 501–4.
8. Elkind, M. M. & H. Sutton. 1960. Radiation response of mammalian cells grown in culture. Radiation Research. **13:** 556–593.
9. Erikson, R. L. & W. Szybalski. Molecular radiobiology of human-cell lines. I. Comparative sensitivity to X rays and ultraviolet light of cells containing halogen-substituted DNA. B. and B. Res. Communications. **4:** 258–261.
10. Hewitt, H. B. & C. W. Wilson. 1959. Brit. J. of Cancer. **13:** 69.
11. Hewitt, H. H. & C. W. Wilson. 1959. Brit. J. of Cancer. **13:** 675.
12. Morkovin, D. & A. Feldman. 1959. Brit. J. Radiol. **32:** 282.
13. Puck, T. T. & P. I. Marcus. 1956. The action of X rays on mammalian cells. I. J. Exptl. Med. **103:** 653–66.
14. Puck, T. T. & D. Morkovin, P. I. Marcus & S. J. Cieciura. 1957. Action of X radiation on mammalian cells. II. J. Exptl. Med. **106:** 485–500.
15. Rubin, H. & H. M. Temin. 1959. A radiological study of cell-virus interaction in the rous sarcoma. Virology. **7:** 75–91.
16. Till, J. E. & E. A. McCulloch. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation Research. **14:** 213–222.
17. Whitmore, G. F., C. P. Stanners, J. E. Till & S. Gulyas. 1961. Nucleic acid synthesis in X irradiated L-strain mouse cells. Biochim et Biophys. Acta. **47:** 66–77.
18. Whitfield, J. F. & R. H. Rixon. 1960. Radiation resistant derivatives of L-cell mouse cells. Exptl. Cell. Research. **19:** 531–538.
19. Whitmore, C. F., J. E. Till, R. B. L. Gwatkin, L. Siminovitch & A. F. Graham. 1958. Increase of cellular constituents in X irradiated mammalian cells. Biochim. et Biophys. Acta. **30:** 583.

RNA AS A PROTECTIVE AGENT AGAINST IRRADIATION OF CELL CULTURES*

Donald E. Rounds
Pasadena Foundation for Medical Research, Pasadena, Calif.

Introduction

The list of chemical agents that can induce a radioprotective response in biological systems has become quite impressive (Patt, 1953; Pihl and Eldjarn, 1958). Of these compounds, interest remains centered around thiols such as cysteine, cysteamine, or aminoethylisothiouronium bromide-hydrobromide (AET).

In recent years, however, postirradiation treatment with nucleic acid preparations was also reported to increase the survival rate of bacteria, rats, and mice given lethal dosages of X or gamma radiation (Cole and Ellis, 1955; Panjevac and Ristic, 1958). Detre and Finch (1958) have reported that a preirradiation injection of yeast extract or yeast ribonucleic acid (RNA) has a similar effect on rats and mice.

Differences in administration time and toxicity levels suggested that radioprotection induced by thiols may be quite different from protection afforded by nucleic acids. This study has attempted to characterize the survival pattern of irradiated cells *in vitro* when pretreated with yeast RNA.

Materials and Methods

RNA concentration. The Fernandes strain of amnion cells was cultured in Eagle's medium with 10 per cent horse serum in miniaturized Rose chambers (Rose, 1954) having an internal diameter of 20 mm. and an internal thickness of 3 mm. In such chambers 3000 to 4000 seeded cells were maintained in the logarithmic phase of growth for a period of 1 week. After 24 hours all cells in a path 1-mm. wide were counted across the diameter of the chambers with the aid of a reticule in the ocular of a phase-contrast microscope.

A total of 40 chambers was treated in a dosage range from 0.1 to 0.5 mg. commercially available yeast RNA per milliliter of medium. Following 24 hours of RNA treatment the cells were recounted, the culture medium was removed, and the cells received 700 r gamma radiation from a cobalt-60 source (47.2 r/min.). Immediately after irradiation the chambers were filled with fresh nutrient fluid and incubated at 37° C. Control cultures with no RNA were similarly handled. Daily counts of cells in all chambers were made for 4 days postirradiation. All counts were recorded as variations from 100 cells at the beginning of the experiment to eliminate error due to unequal seeding of the chambers.

Duration of RNA treatment. A total of 64 T30 flasks was seeded with 300,000 amnion cells per flask. After a 24-hour incubation period, one half of the cultures was treated with 0.1 mg./ml. yeast RNA for periods ranging from 3

* The work described in this paper was supported in part by Air Force Contract AF 41(657)-337 under the sponsorship of The School of Aviation Medicine, United States Air Force Aerospace Medical Center, Air Training Command, Brooks Air Force Base, Tex.

to 24 hours. An equal number of control flasks was kept for equivalent periods of time in Eagle's medium. Following the treatment period all flasks were irradiated with 700 r gamma radiation in an air phase and incubated for 4 days. The cells from each flask were then trypsinized, washed, suspended in saline, and enumerated with the aid of a Coulter cell counter.

Variation of radiation dose. Amnion cells were subcultured into 96 T30 flasks using 300,000 cells per flask. The elements in 6 flasks for each gamma dose were treated for 24 hours with 0.1 mg./ml. yeast RNA, while cultures in 6 control flasks were maintained for an equal length of time in Eagle's medium. The treated and control flasks were radiated with a gamma dosage range of 0

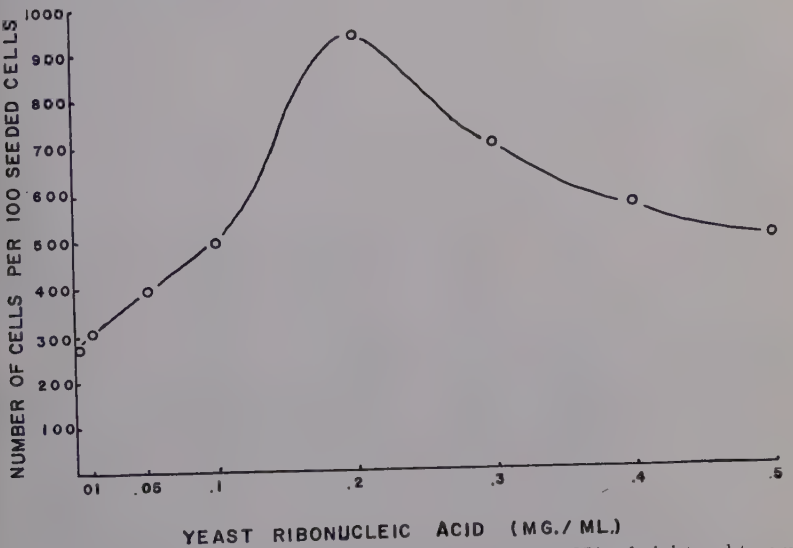


FIGURE 1. The effect of increasing concentrations of yeast RNA administered to amnion cells 24 hours prior to 700 r gamma radiation from a cobalt-60 source.

to 700 r. The cultures were incubated for 4 days postirradiation, and the surviving cell population for each flask was evaluated with a Coulter cell counter.

Response of different cell types. Seven different cell strains were seeded into modified Rose chambers and treated as described above. These cells types consisted of Fernandes amnion, Chang conjunctiva and liver, Detroit-98 and Detroit-6 human bone marrow (Berman and Stulberg, 1956), HeLa, and KB. Each cell line contained in 10 or more chambers was treated for 24 hours with 0.1 mg./ml. yeast RNA prior to 700 r gamma radiation, while cells in an equal number of chambers served as untreated irradiated controls. The average number of cells per 100 seeded cells was plotted for the fourth day after irradiation.

Results

Preirradiation treatment of amnion with yeast RNA resulted in increased numbers of surviving cells. The degree of survival was proportional to the

RNA concentration to an optimum of 0.2 mg./ml. (FIGURE 1). Decreasing survival with RNA doses between 0.3 and 0.5 mg./ml. suggested that a balance between radioprotection and RNA toxicity was becoming manifest.

The data presented in FIGURE 2 suggested that a treatment period of 3 to 6 hours with yeast RNA does not elicit a protective response. Amnion cells showed protection only after 8 to 10 hours of treatment. A maximum population increase was observed at 12 and 24 hours' exposure to RNA.

Exposure of RNA-treated amnion cultures to increasing gamma-ray doses revealed a rate of cell survival that was elevated but parallel to the survival curve of untreated irradiated control cells (FIGURE 3). Since the slopes of the two curves were approximately the same, the data suggested that the

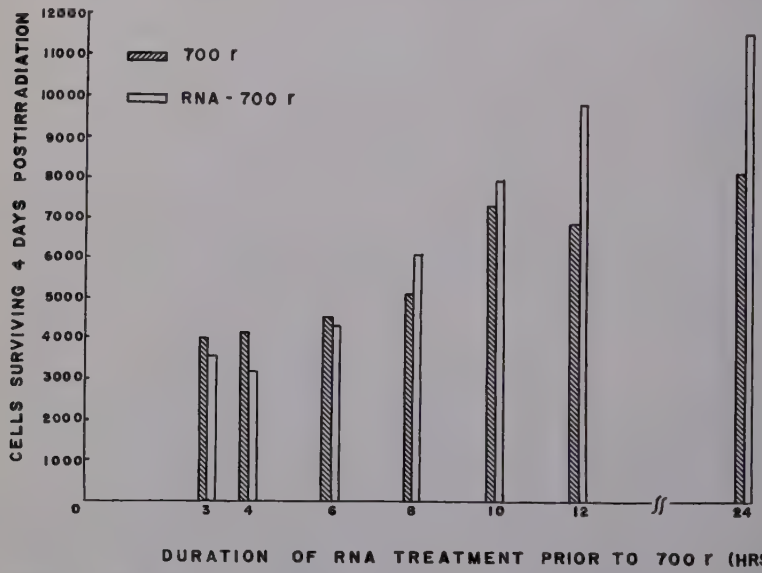


FIGURE 2. The effect of 3- to 24-hour treatment periods using 0.1 mg./ml. yeast RNA prior to 700 r gamma radiation on the survival rate of amnion cells. The shaded bars represent untreated controls; the open bars represent the RNA-treated cell populations.

radiosensitivity of the cells was unchanged although the cell population was increased by a uniform amount.

Daily counts of amnion cells exposed to 700 r showed that growth of untreated, irradiated cultures was inhibited within a 24-hour period (FIGURE 4). RNA-treated cultures, however, exhibited a growth rate of unirradiated cultures. Thus RNA treatment appeared to delay radiation damage for a 2-day period, permitting an increase in the cell population as compared with the untreated, irradiated control cultures.

Different cell lines responded differently to yeast RNA followed by irradiation. Increased numbers of surviving cells were observed in the 4 cell types derived from nonmalignant tissues (FIGURE 5), namely human bone marrow (Detroit-98), liver, amnion, and conjunctival elements. Cultures of 3 established strains that have been considered malignant showed no protective response. These included HeLa, KB, and bone-marrow (Detroit-6) types obtained from an individual with a malignancy.

Discussion

Increased survival of amnion cells derived from a preirradiation treatment with yeast RNA appeared to result from a mechanism quite different from the protection afforded by cysteine or other thiols. For example Bases (1959) has demonstrated that HeLa cells showed increased survival with a preirradiation

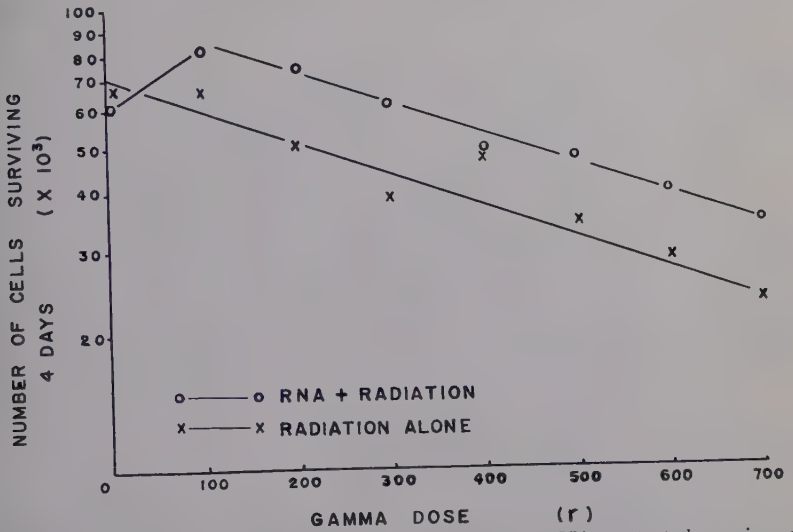


FIGURE 3. Dose-response relationship for control and RNA protected amnion cells. Yeast RNA was added 24 hours before gamma radiation at a concentration of 0.1 mg./ml.

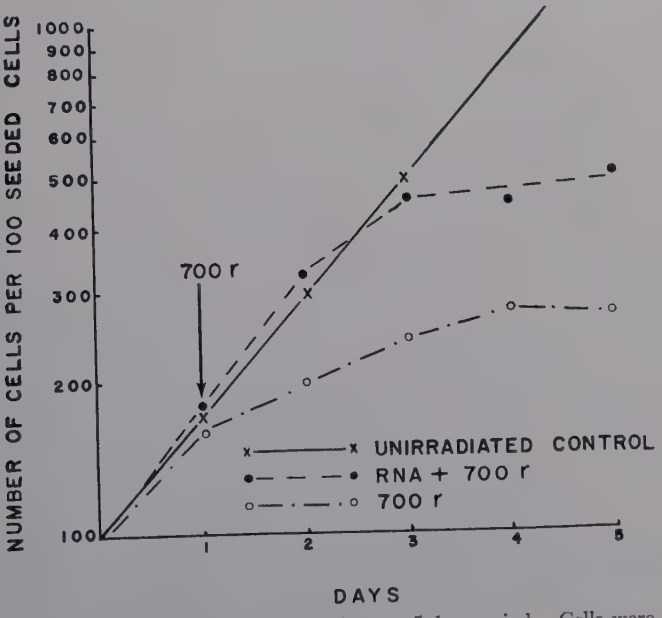


FIGURE 4. Amnion cell population throughout a 5-day period. Cells were radiated on day 1. Treated cultures were given 0.1 mg./ml. yeast RNA for 24 hours prior to irradiation.

treatment with AET of only 15 min. A similar response was observed by Patt *et al.* (1952) using cysteine as a protective agent for rabbit thymocytes. The data in FIGURE 2 indicated that amnion cells required an 8 to 10 hour exposure to RNA before a protective response could be measured. This prolonged time lag suggested that the amnion tissue may be hydrolyzing and resynthesizing other products from the yeast RNA. Such a hypothesis is currently being explored.

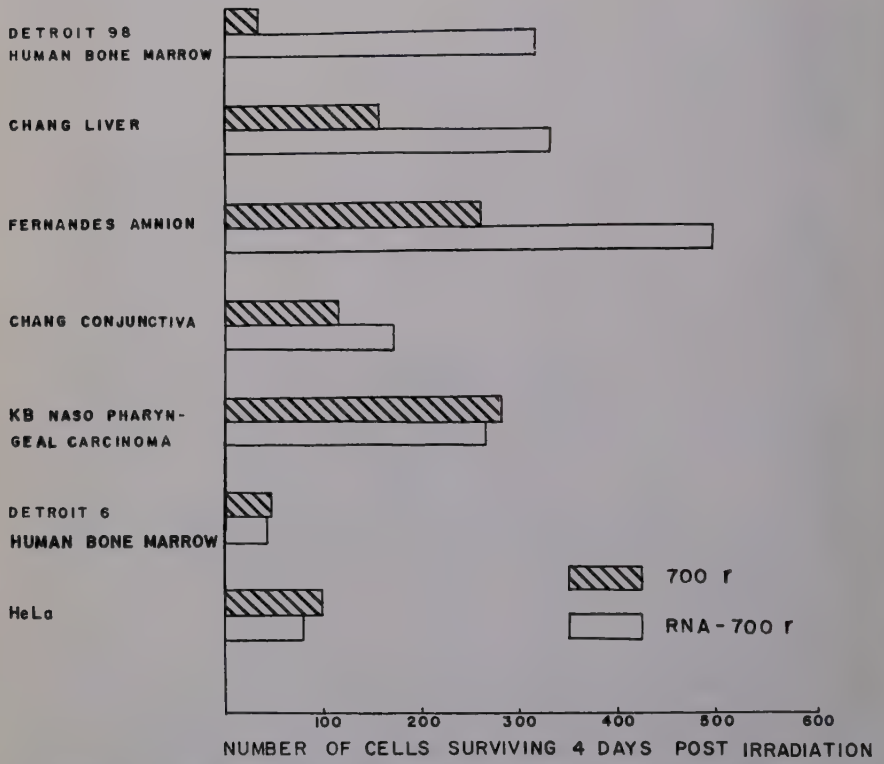


FIGURE 5. Populations of different established cell strains surviving 4 days postirradiation. Shaded bars represent untreated, radiated controls. Open bars represent cultures exposed to 0.1 mg./ml. yeast RNA for 24 hours prior to gamma radiation.

The response of a biological system to increasing radiation dosages with and without factors producing anoxia (Patt *et al.*, 1952; Rounds *et al.*, 1959) has been expressed by curves originating from a common source and showing a continual divergence. The pattern shown in FIGURE 3 consisted of parallel rather than diverging lines. The data expressed by FIGURES 3 and 4 suggested that radiation damage was delayed for 48 hours in RNA-treated cultures, during which time the cell population increased at a normal rate of growth. It is interesting to note from the data in FIGURE 4 that yeast RNA did not stimulate growth of the amnion tissue during the 24-hour treatment period before irradiation. Thus an increase in cell number was not due to growth stimulation.

Storaasli *et al.* (1953) and Patt (1958) have reported that protection with cysteine is not confined to normal tissue, but that radioresistance of tumor tissue may also be enhanced. In contrast the effect of yeast RNA may result in a protective activity associated only with nonmalignant tissues (FIGURE 5). If the RNA is partially hydrolyzed, as previously suggested, the findings of Bennett *et al.* (1959) may be pertinent to this problem. These authors have reported that malignant tissues fail to utilize preformed nucleotides to the same extent as nonmalignant cells.

Foley and Handler (1957) have demonstrated differences in the tumor-forming capacity of established cell strains derived from malignant or nonmalignant sources. The data in FIGURE 5 suggest another criterion for establishing the degree of malignancy of cell strains *in vitro*. It is interesting to note that the two bone-marrow strains showed significant differences in their response to radiation. The Detroit-6 strain, while not derived from a primary malignancy, was obtained from an individual with a malignant growth (Berman and Stulberg, 1956). No such malignancy was detectable in the source of the Detroit-98 bone-marrow tissue. Thus, in addition to potential clinical applications, RNA treatment of biological systems followed by radiation offers a possible test method to elucidate biochemical differences between normal and malignant tissue *in vitro*.

Summary

The Fernandes strain of amnion cells, treated with dosages ranging from 0.01 to 0.5 mg./ml. of yeast RNA 24 hours prior to 700 r gamma radiation, showed increased survival as compared with control cultures not so treated. Approximately 3 times as many cells survived 700 r when pretreated with 0.2 mg./ml. yeast RNA.

A minimum RNA treatment time of 8 to 10 hours was necessary before a protective response could be elicited in irradiated amnion cells. Thus protection may result only after the yeast RNA is utilized by the cell.

RNA-treated and control cultures of amnion cells were exposed to gamma doses ranging from 0 to 700 r. Survival was represented by parallel lines on a semilogarithmic plot, which suggested a possible increase in the cell number of treated cultures prior to the onset of radiation damage. Daily counts of amnion cells indicated that RNA-treated elements multiply at a normal rate for 2 days postirradiation, whereas untreated irradiated cultures showed an immediate inhibition in the rate of growth.

Seven different established cell strains exhibited variations in the response of preirradiation treatment with yeast RNA. Four cell types of nonmalignant origin showed from 0.5 to 8 times more cells surviving 700 r when pretreated with RNA than untreated, irradiated cultures. Three cell lines of malignant origin showed no evidence of protection.

References

BASES, R. E. 1959. Some applications of tissue culture methods to radiation research. Cancer Research. **19:** 311–315.

BENNETT, L. L., JR., H. E. SKIPPER, D. SMITHERS & E. H. HAYES. 1959. Searches for exploitable biochemical differences between normal and cancer cells. IV. Utilization of nucleosides and nucleotides. Cancer Research. **19:** 217–221.

Berman, L. & C. S. Stulberg. 1956. Eight culture strains (Detroit) of human epithelial-like cells. Proc. Soc. Exptl. Biol. Med. **92:** 730–735.

Cole, L. J. & M. E. Ellis. 1955. Radiation recovery activity associated with a deoxyribonucleoprotein fraction from mouse spleen. Federation Proc. **14:** 29.

Detre, K. K. & S. C. Finch. 1958. Radiation protective effects of yeast extract and yeast ribonucleic acid. Science. **128:** 656–657.

Foley, G. E. & A. H. Handler. 1957. Differentiation of "normal" and neoplastic cells maintained in tissue culture by implantation into normal hamsters. Proc. Soc. Exptl. Biol. Med. **94:** 661–664.

Panjevac, B. & G. Ristic. 1958. Recovery from radiation injury in rats following administration of rat isologous highly polymerized nucleic acids. Bull. Inst. Nuclear Sci. (Belgrade, Yugoslavia). **8:** 159–160.

Patt, H. M., M. E. Blackford & R. L. Straube. 1952. Effect of X rays on thymocytes and its modification by cysteine. Proc. Soc. Exptl. Biol. Med. **80:** 92–97.

Patt, H. M. 1953. Protective mechanisms in ionizing radiation injury. Phys. Rev. **33:** 35–76.

Patt, H. M. 1958. Modification of radiation effects by chemical means. *In* Progress in Radiation Therapy. **:** 115–127. F. Buschke, Ed. New York, N.Y.

Pihl, A. & L. Eldjarn. 1958. Pharmacological aspects of ionizing radiation and of chemical protection in mammals. Pharmacol. Rev. **10:** 437–474.

Rose, G. 1954. A separable and multipurpose tissue culture chamber. Texas Rept. Biol. and Med. **12:** 1074–1083.

Rounds, D. E., C. M. Pomerat, L. C. Logie, Y. H. Nakanishi & D. Anderson. 1959. Irradiation of cells in tissue culture. VI. The influence of oxygen injury to cells by gamma irradiation from a cobalt-60 source. Z. Zellforsch. **50:** 425–432.

Storaasli, J. P., S. A. Rosenberg & H. L. Friedell. 1953. Effect of cysteine on radiosensitivity of lymphosarcoma. Cancer. **6:** 1244–1247.

QUANTITATIVE STUDIES OF RADIATION EFFECTS ON CELL REPRODUCTIVE CAPACITY IN A MAMMALIAN TRANSPLANTABLE TUMOR SYSTEM *IN VIVO*

Roger J. Berry and J. Robert Andrews
Radiation Branch, National Cancer Institute, Public Health Service, Bethesda, Md.

The Radiation Branch of the National Cancer Institute has long been identified with attempts to replace qualitative observations with quantitative data in the study of the biological effects of ionizing radiation. Hollcroft *et al.*, in this laboratory in 1952 provided evidence of the effects of oxygenation and anoxia on the response of a transplanted murine lymphosarcoma to X irradiation, using the best quantitative methods available at that time.[1] It was not, however, until Puck and his associates[2,3] developed the techniques of clonal culture that the effects of ionizing radiation upon the reproductive capacity of mammalian cells could be studied directly, and the recent studies of Elkind and Sutton[4] at the National Cancer Institute reflect further fruitful utilization of these quantitative *in vitro* methods. The experiments of Hewitt and Wilson demonstrated that conditions obtaining in the artificially controlled environment of the free-cell culture in test tubes could be simulated in the homeostatically controlled environment of the living host and that the response of mammalian cells to ionizing radiation appeared to be similar *in vitro* and *in vivo*.[5,6,7,8]

At the National Cancer Institute we have been most fortunate in having the ascites form of the P-388 lymphocytic leukemia in the DBA/2 mouse strain, and the ability of leukemic cells from the ascitic fluid of mice bearing this transplanted tumor to produce leukemia in recipient mice has been used as a measure of cell reproductive capacity. In the present discussion, we shall outline the system as used and demonstrate its freedom from complicating factors of host immunity; we shall present the dose response to X rays obtained under various conditions of oxygenation and compare the effects of single and fractionated X-ray doses. Preliminary data on the ability of halogenated pyrimidine deoxyribosides (halogenated DNA precursors) to modify the response of mammalian cells to irradiation *in vivo* will be discussed.

Materials and Methods

Mice. Strain DBA/2JN female mice were used exclusively throughout this study, and were three months old at the start of each experiment. They were supplied by the Animal Production Section, Division of Research Services, National Institutes of Health, from a brother-sister mated colony.

Tumor. The P-388 lymphocytic leukemia was obtained from M. Potter, National Cancer Institute, at the 143rd passage and has since been carried through over 130 serial weekly passages in this laboratory. The tumor arose in a methylcholanthrene-painted DBA/2 female mouse in June 1955 and was converted to the ascitic form at the first passage. Tumor generations 245–270 were used in the experiments reported here, but no change in either the character of the tumor or the mean time elapsed between inoculation and death

has been noted at any time. The ascites is virtually bloodless and contains approximately 10^8 tumor cells per ml. on the seventh day. For routine passage, 1 to 1.5 ml. of ascitic fluid from a 7-day tumor-bearing mouse was aspirated by syringe under sterile conditions and immediately diluted with an equal volume of ice-cold Tyrode's solution. A 1:80 dilution of this suspension was made for counting by phase microscopy (although one of the simpler white blood cell counting methods would have sufficed). The characteristic round cells with bright halos under the phase microscope were considered to be morphologically intact tumor cells; these numbered over 90 per cent of the total of cells seen in any suspension and thus minimized possible errors of counting due to mistaken cell identification. The counted cell suspension was diluted with ice-cold Tyrode's solution to a concentration of 10^7 cells/ml. and 0.1 ml. was injected intraperitoneally into each of 6–8 mice.

Titration of leukemia cells. Serial twofold or fourfold dilutions of counted tumor cell-Tyrode's suspensions were prepared as above; 0.1 ml. aliquots of each of a series of 4–6 such dilutions were injected intraperitoneally into groups of 5–6 mice. The mean number of leukemic cells injected into any group of mice was calculated by multiplying the cell count for the initial suspension by the dilution factor. The mice were then observed for 40 days and the number dying per group was recorded. All animals that died within the 40-day experimental period were inspected for gross pathologic evidence of leukemia, and those dying from other causes were excluded from the data (in fact, only 3 animals have died from causes other than their transplanted leukemia in the first 20 experiments). Animals were continuously observed for up to 120 days in early experiments, but the fact that no additional leukemias had developed after 40 days led to the curtailment of the experimental period. From the mortality data, the number of cells required to produce leukemia "takes" in 50 per cent of a group of recipient mice (the TD-50) was calculated by graphic interpolation, using the accumulation method of Reed and Muench.[9] The range of numbers of tumor cells injected in any experiment was selected on the basis of the predicted TD-50; every experimental titration included groups in which no deaths occurred and other groups with 100 per cent mortality to allow reliable interpolation in calculating the TD-50 values.

Irradiation. X rays were generated in the gold target of a Van de Graaff generator operating at 3 Mev with a beam HVL of 10.4 mm. Pb. Tumor-bearing animals were irradiated total-body through the back at 50 to 100 cm. TSD at dose-rates of 800 to 3200 rpm. Exposure dose was measured using a Bendix pocket ionization chamber and checked with ferrous sulfate; absorbed dose in rads was calculated using the factor 97 erg/gm./roentgen for 3 Mev incident beam, and the dose inhomogeneity through the abdomen of the irradiated mouse was less than 10 per cent.

Results

The TD-50 for leukemic cells from unirradiated donor mice averaged 2.3 (S.D. $\pm$ 0.6) in 16 experiments. The TD-50 was not affected by inoculation of 1–4 viable cells in the presence of 10^6 radiation-killed cells, nor was it elevated by pre-inoculation of recipient mice with 10^6 radiation-killed cells, thus giving

evidence that there is no significant immune incompatibility between tumor and host.

Dose-response curve. Tumor-bearing donor animals were subjected to a single dose of total-body irradiation immediately prior to aspiration of the ascitic fluid. The "surviving fraction" (cells remaining capable of reproduction) after irradiation is defined as: control TD-50/irradiated TD-50, and is a function of X ray dose. The results are shown in FIGURE 1. There was no difference noted between results in experiments in which the animals were breathing air

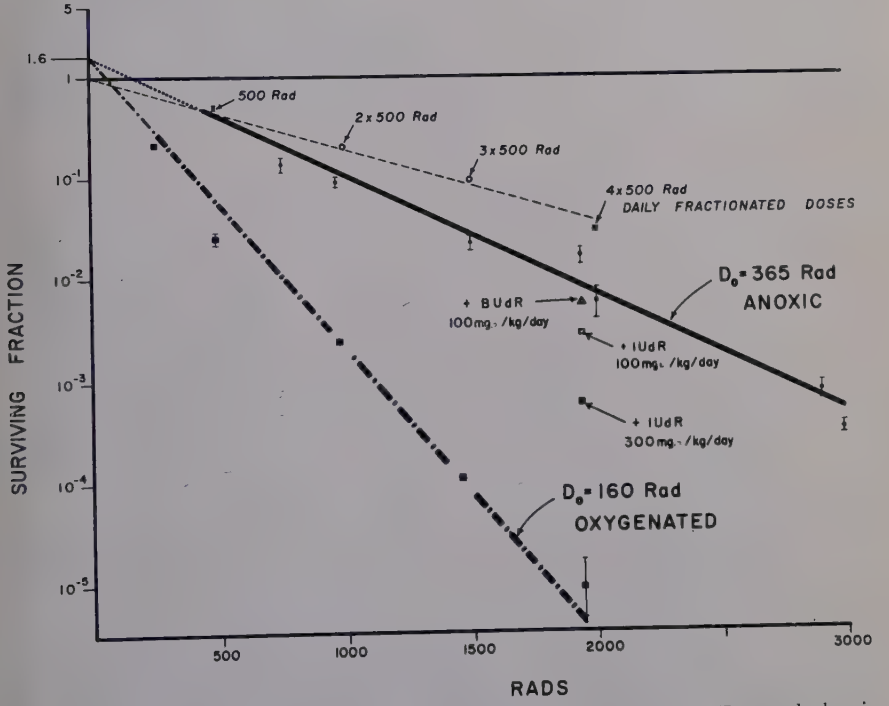


FIGURE 1. Dose-response, single and fractionated irradiation, 3 Mev X rays, leukemia P-388. Key: ——, anoxic; ·-·-·-·-·, oxygenated; – – –, daily fractionated doses.

or 100 per cent oxygen during the time of irradiation and those in which the donor animal had been killed 10 to 20 min. prior to irradiation. Results under all these experimental conditions therefore represent the ANOXIC radiosensitivity of reproductive capacity in P-388 leukemic cells. Each point represents the mean of 2 to 5 separate experiments, and the standard error is shown. The regression line was determined by the method of least squares and the slope of the straight portion indicates a mean lethal dose (D_0) of 365 rads and an extrapolation number greater than one and approximating two under these anoxic conditions.

In order to assess the effect of radiation upon reproductive capacity of the P-388 leukemic cells when irradiated *in vivo* under oxygenated conditions, 3 per

cent hydrogen peroxide was injected intraperitoneally to a final concentration of 0.2 *M* in the ascitic fluid of tumor-bearing donor animals immediately prior to irradiation. This procedure did not raise the TD-50 of unirradiated cells in six identically repeated experiments (TD-50 = 2.2 ± 0.5), but the profuse liberation of oxygen bubbles by the action of tumor fluid catalase provided adequate oxygenation for the tumor cells during irradiation. The dose response under these conditions is also shown in FIGURE 1; the slope of the straight portion differs from the anoxic curve by 2.3:1 (the "oxygen effect ratio"), with a D_0 of 160 rads and an extrapolation number similar to that of the anoxic curve.

Dose fractionation. To compare the above results of single-dose irradiation with effects of the same total dose delivered in multiple fractions, tumor-bearing donor animals were given 1–4 daily total-body exposures of 500, 1000, or

TABLE 1
DOSE FRACTIONATION, LEUKEMIA P-388

	TD-50	Surviving fraction
Control	2.3	1.0
1000 Rads	23	0.1
2 × 500 Rads	11.5	0.2
1500 Rads	88.5	0.026
3 × 500 Rads	26	0.089
2000 Rads	330	0.007
2 × 1000 Rads	139	0.017
4 × 500 Rads	82	0.028
3000 Rads	5000	0.00046
2 × 1500 Rads	2420	0.00095
3 × 1000 Rads	535	0.0043

1500 rads. The 24-hour separation of X-ray doses exceeds the 15-hour doubling time of the tumor, which was determined by plotting the mean survival of large groups of animals following tumor inocula of $1 - 1 \times 10^6$ cells. The final dose was given immediately prior to aspiration of the ascitic fluid and titration into recipient animals. The results are shown in TABLE 1; as the same total dose is divided into greater numbers of fractions, there is proportionate increase in the percentage of tumor cells remaining capable of reproduction. No increase in the percentage of morphologically damaged cells was noted in the cell suspensions from animals which had received fractionated doses, and the concentration of leukemic cells in the ascitic fluid was significantly lowered (> 20 per cent) only in the 3 × 1000 rads and 4 × 500 rads groups. The data for multiple 500 rads daily fractions are also shown in FIGURE 1; that repeated cycles of recovery of sublethally irradiated cells have occurred is immediately apparent, and the extrapolation of the line passing through these points passes also through the origin.

Modification of radiation response with pharmacologic agents. Tumor-bearing

donor animals were given 4 daily intraperitoneal injections of either 5-bromodeoxyuridine (BUdR) or 5-iododeoxyuridine (IUdR) suspended in Tyrode's solution. Doses of 100 to 300 mg./kg. body weight/day were used, the total volume of fluid injected per day was 0.2 ml. per mouse, and the final dose of the drug was given 24 hours prior to irradiation and/or aspiration of the ascitic fluid for titration to determine the TD-50. The results are shown in TABLE 2. Administration of IUdR alone did not affect the TD-50 for leukemic cells from unirradiated donor mice either at 100 or 300 mg./kg./day dose levels. At both drug doses used, however, there was potentiation of the radiation effect and decrease in the surviving fraction following 1940 rads X rays, with the magnitude of potentiation apparently dependent upon drug dosage in the range used here. The results with BUdR indicate that a dose of 300 mg./kg./day × 4 doubled the TD-50 for cells from unirradiated donors in 2 experiments, while

TABLE 2

DRUG POTENTIATION OF RADIATION EFFECT, LEUKEMIA P-388

	TD-50	Surviving fraction
5-Iododeoxyuridine (IUdR)		
Control	2.8	
Control + IUdR 100 mg./kg./day	2.3	
Control + IUdR 300 mg./kg./day	1.8	
1940 rads	170	0.016
1940 rads + IUdR 100 mg./kg./day	840	0.0027
1940 rads + IUdR 300 mg./kg./day	3200	0.00056
5-Bromodeoxyuridine (BUdR)		
Control	1.5–1.7	
Control + BUdR 100 mg./kg./day	1.6	
Control + BUdR 300 mg./kg./day	3.0–3.5	
1940 Rads	123	0.013
1940 Rads + BUdR 100 mg./kg./day	280	0.0057
1940 Rads + BUdR 300 mg./kg./day	350	0.0093

100 mg./kg./day failed to alter the TD-50 values observed. At the lower dosage of the drug, slight potentiation of the effect of 1940 rads X rays was seen, but although the TD-50 values following irradiation are elevated at the higher drug dose, any potentiation of the radiation effect is masked by the direct toxic effect of the BUdR upon the leukemic cells and the surviving fraction is essentially unaltered. Results of these experiments are also plotted in FIGURE 1 for graphic comparison.

Discussion

The P-388 transplantable murine leukemia offers an *in vivo* system for radiobiologic studies with many of the advantages heretofore attained only *in vitro*. This tumor has been maintained in females of the closely inbred strain in which it arose, and the remarkable genetic constancy it has shown in 270 serial passages may be due in part to rigid standardization of routine transplantation schedule and technique. The fact that the tumor has never been noted to regress spontaneously, plus the repeatably low TD-50 and inability to alter

the TD-50 value, either by giving small numbers of viable tumor cells in the presence of large numbers of radiation-killed cells, or by pretreatment with large numbers of radiation-killed cells, satisfies Revesz' stringent criteria for absence of significant host-tumor immune incompatibility.[11]

The dose-response curves for P-388 leukemic cells under oxygenated and anoxic conditions agree remarkably well with the observations of Hewitt and Wilson upon murine leukemic cells derived from the liver, and with the *in vitro* observations of Puck *et al.*[12] in several lines of human and hamster-derived cells of normal or malignant origin when corrected for errors in dosimetry. It is not surprising that a majority of the tumor cells in the ascitic fluid are anoxic whether the animal is breathing air or 100 per cent oxygen, although theoretical calculations by Hewitt and Wilson[7] suggest that it would be difficult indeed to detect the presence of a small number of oxygenated cells in the presence of a large predominance of anoxic cells. The tumor volume in the seventh-day mouse averages 5 to 7 ml., rendering it unlikely that at any point in time a majority of cells could be within the 145 μ distance from a blood capillary which Gray and Thomlinson have shown to be the maximum radius of oxygenation in tissue.[13] The use of hydrogen peroxide to oxygenate ascites tumor has previously been shown by Deschner and Gray[14] to produce no increase in anaphase abnormalities with the drug alone at 0.1 *M* concentration, while simulating irradiation results obtained *in vitro* under equilibration with 100 per cent oxygen. The results in the present system indicate that a 0.2 *M* concentration of hydrogen peroxide alone did not affect the reproductive capacity of leukemic cells, but the release of oxygen bubbles by the action of tumor fluid catalase provided adequate oxygenation during irradiation at the high dose rates and short exposure times used.

The data obtained following fractionated irradiation confirm *in vivo* the demonstration *in vitro* of recovery in sublethally irradiated cells recently reported by Elkind and Sutton.[4] The results reported here were obtained in an anoxic system, whereas Elkind used oxygenated cell suspensions, suggesting that the mechanism of recovery is independent of oxygen tension and that destruction of reproductive capacity is contingent only upon the accumulation within the cell of the requisite number of ionization events (hits). While cell death in tumors receiving fractionated irradiation cannot be ruled out and further study of the kinetics of the irradiated tumor population are needed, the failure to find an increase in the number of morphologically damaged cells and the maintenance of normal tumor concentration (cells per milliliter) up to four fractionated doses support the contention that these data represent true recovery of sublethally irradiated cells *in vivo*.

The potentiation of the radiation response by pretreatment of tumor-bearing animals with halogenated pyrimidine deoxyribosides represents only a preliminary study but illustrates clearly the usefulness of the *in vivo* system described here. Djordjevic and Szybalski recently reported that mammalian cells grown *in vitro* in either IUdR or BUdR were more sensitive to reproductive sterilization by ultraviolet light and X rays.[15] Results obtained in a system such as reported here are more likely to reflect clinical usefulness in tumor therapy as the problems of drug incorporation, route of administration, and

toxicity can be quantitatively and simultaneously studied. Further investigation of modification of the radiation effect by pharmacologic agents is currently in progress in our laboratory, as are studies of the comparative effects of radiations of different ionization densities (LET).[16,17]

Summary

The transplantability of leukemic ascites cells from mice bearing the transplanted lymphocytic leukemia P-388 has been utilized as a measure of cell reproductive capacity. The response of mammalian cells to ionizing radiation appears to be similar *in vivo* and *in vitro*. The system is relatively free of host-immunity factors and has been used to study: (1) the effects of oxygenation and anoxia, (2) the effects of dose fractionation and aspects of cellular recovery, and (3) changes in radiation response induced by pharmacological agents.

Acknowlegments

Thanks are due to Marion Matthews and Bruce Weed for devoted and invaluable assistance.

References

1. Hollcroft, J. W., E. Lorenz & M. Matthews. 1952. Factors modifying the effect of X-irradiation on regression of a transplanted lymphosarcoma. J. Nat. Cancer Inst. **12:** 751–763.
2. Puck, T. T. & P. I. Marcus. 1956. Action of x-rays on mammalian cells. J. Exptl. Med. **103:** 653–666.
3. Puck, T. T., D. Morkovin, P. I. Marcus & S. J. Cieciura. 1957. Action of x-rays on mammalian cells. II. Survival curves of cells from normal human tissues. J. Exptl. Med. **106:** 485–500.
4. Elkind, M. M. & H. Sutton. 1960. Radiation response of mammalian cells grown in culture. I. Repair of x-ray damage in surviving chinese hamster cells. Radiation Research. **13:** 556–593.
5. Hewitt, H. B. 1958. Studies of the dissemination and quantitative transplantation of a lymphocytic leukemia of CBA mice. Brit. J. Cancer. **12:** 378–401.
6. Hewitt, H. B. & C. W. Wilson. 1959. A survival curve for mammalian leukemia cells irradiated *in vivo* (Implications for the treatment of mouse leukemia by whole-body irradiation). *Ibid.* **13:** 69–75.
7. *Idem.* 1959. The effect of tissue oxygen tension on the radiosensitivity of leukemia cells irradiated *in situ* in the livers of leukemic mice. *Ibid.* **13:** 675–684.
8. *Idem.* 1960. Further studies relating to the implications of radiation survival curve data for the treatment of CBA mouse leukemia by whole-body irradiation. *Ibid.* **14:** 186–194.
9. Reed, L. J. & H. Muench. 1938. A simple method for estimating fifty per cent points. Am. J. Hyg. **27:** 493–497.
10. Handbook 78, U.S. Dept. of Commerce, National Bureau of Standards. 1961. Report of the international commission on radiological units and measurements (ICRU). U.S. Printing Office. Table 8.1. : 30.
11. Revesz, L. 1960. Detection of antigenic differences in isologous host-tumor systems by pre-treatment with heavily irradiated tumor cells. Cancer Research. **20:** 443–451.
12. Morkovin, D. & A. Feldman. 1959. Dosimetry in living cells irradiated on glass. A correction. Brit. J. Radiol. **32:** 282.
13. Thomlinson, R. H. & L. H. Gray. 1955. Histological structure of some human lung cancers and possible implications for radiotherapy. Brit. J. Cancer. **9:** 539–549.
14. Deschner, E. E. & L. H. Gray. 1959. Influence of oxygen tension on x-ray induced chromosomal damage in Ehrlich ascites tumor cells irradiated *in vivo* and *in vitro*. Radiation Research. **11:** 115–146.
15. Djordjevic, B. & W. Szybalski. 1960. Genetics of human cell lines. III. Incorpora-

tion of 5-bromo- and 5 iododeoxyuridine into the deoxyribonucleic acid of human cells and its effect on radiation sensitivity. J. Exptl. Med. **112:** 509–531.

16. Berry, R. J. & J. R. Andrews. 1961. Modification of the radiation effect upon the reproductive capacity of tumor cells *in vivo* with pharmacological agents. (Abstract.) Radiation Research. **14:** 447.
17. Andrews, J. R. & R. J. Berry. 1961. The relative biological effectiveness (RBE) of neutron irradiation of a quantitative *in vivo* mouse tumor cell system. (Abstract.) Radiation Research. **14:** 452.

STUDIES ON RADIOPROTECTION OF VARIOUS COMPOUNDS *IN VITRO* AND *IN VIVO*

Annelies Schleich

Institut für experimentelle Krebsforschung, University of Heidelberg, Heidelberg, West Germany

During the past 30 years considerable research has been going on in the field of radiation protection, mostly in the way of animal experiments. Tissue culture experiments have been added enabling us to differentiate between the direct and indirect protective effect.[1]

In our laboratory my associates and I have tested the radioprotective capacities of a number of compounds. The results have been measured on the survival rate of white mice and in tissue culture. W. Sachsenmaier of the biochemical laboratory of our institute has carried out a series of tests to determine the change in UV absorption of thymine solutions after irradiation. He has also measured the change in the viscosity of the DNA following irradiation. I am grateful to him for being able to present also some of his results, together with those of our own biological studies.

Material and Methods

Pen-bred white stock mice have been used for the *in vivo* experiments. The compounds were injected intraperitoneally immediately before irradiation unless otherwise specified.

The *in vitro* experiments were carried out with strain AFi, a human fibrosarcoma of the humerus isolated in 1938 in G. O. Gey's laboratory at the Johns Hopkins Hospital, Baltimore.[2] The cells were grown in roller tubes with a fluid medium consisting of 6 parts of Hanks' BSS and 4 parts of human placental cord serum. The medium was changed twice a week. Experiments for fixation and staining were performed on so-called flying tube slips with 4 colonies on each of them. The long-term cultures were grown on the tube wall, at 10 rph, again 4 colonies each, in rotating drums. Groups of 3–4 roller tubes have been used for each count. For histological evaluation the cell cultures were fixed with Bouin's solution and stained with Mayer's hemalum. One thousand cells per tube slip have been counted systematically through serial zones of outgrowth. The compounds were added to the medium at a tenfold concentration in Hanks' BSS. The X-ray controls received the same amount of Hanks' BSS.

X-ray source was the Siemens Dermopan 50 kv., at 25 mAmp., aluminum filter, 1.0 mm., 30 cm. target distance, delivering 60 rpm. X-ray measurements were taken with the Simplex universal dosimeter.

It was our object to extrapolate our observations over a long period of time without disturbing the cell population, especially through transfers; consequently we chose a strain in continuous culture. For this we used strain AFi, the fibrosarcoma strain maintained during the last 23 years in continuous culture. We have found it very stable in its growth rate and in its mitotic behavior. It can be transferred in very small colonies and kept up to 4 weeks

in the same roller tube without transfer. The growth appears as a monolayer and is especially useful for histological purposes. The LD_{100} of this strain is approximately 700 r. FIGURE 1 gives a picture of the strain AFi control. Changes occurring during 11 days following irradiation with 700 r are shown in FIGURE 2.

When we started out with our experiments using animals and tissue culture we tried in general to set up conditions as similar as possible, especially as

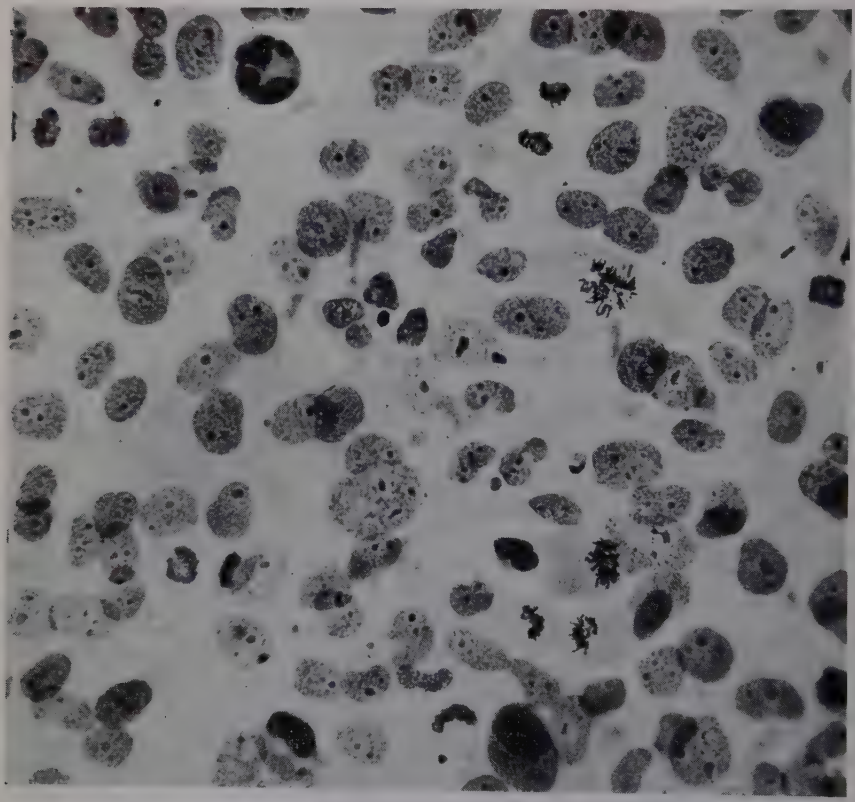


FIGURE 1. Human fibrosarcoma strain AFi, control culture. Mayer's hemalum. ×420.

regards the timing of the chemical treatment and the irradiation. Mice were injected immediately before irradiation. The cells *in vitro* were immersed in the experimental solution for 10 min. during incubation and then irradiated. Immediately after irradiation the medium was removed and replaced by the usual growth medium of 6 parts of Hanks' BSS and 4 parts of human placental cord serum.

Some Results

Our diagrams show first the results of our experiments with white mice. The results in tissue culture follow. Here we plotted the number of mitoses

against the multinucleated cells. This gave us an index of irradiation damage in relation to the mitotic activity.

With the penicillamine (FIGURE 3*a*) we did not see any protective effect but rather a tendency to sensitization of the animals to radiation. In tissue culture examinations (FIGURE 4*a*) we found no remarkable difference between the controls and the penicillamine-treated cultures. The long-term growth gives a similar picture to the animal experiment.

Histamine (FIGURE 3*b*) gave rather hopeful results *in vivo*, but *in vitro* (FIGURE 4*b*) no protection was found.

Kinetin (FIGURE 3*c*) in the animal, given once immediately before the irradiation and in other experiments also on several days following irradiation produced no protection.

In tissue culture (FIGURE 4*c*) the number of the multinucleated cells was as high as in the control cultures. The number of mitoses showed no increase. The long-term cultures died shortly before the controls.[3,4,5]

Thiourea (FIGURE 3*d*) generally is known as a protecting agent. The sarcoma cultures (FIGURE 4*d*) responded with a definite decrease in the multinucleated cells during the first days. The result in continuous cultures was not definite.

Thioctic acid (FIGURE 3*e*) gave no protection in the animal; however in tissue culture (FIGURE 4*e*) the sarcoma cells showed a reduction in the number of multinucleated cells, whether treated continuously or given in a single dose. The long-term cultures did not survive significantly during the continuous treatment.[6]

The best protective results that we have obtained *in vivo* and *in vitro* were given by Cysteinamine (FIGURE 3*f*). For our experiments we used the cysteinamine base $NH_2CH_2CH_2SH$ (2-amino-ethanthiol, 2-mercaptoethylamin) with a *p*H of 9.0 which was tolerated by the animals and the cultured cells (FIGURE 4*f*). Here we see a very positive protective effect. In the controls the multinucleated cells have a peak already during the third day while the treated cells do not follow until the eighth day. The mitotic activity in the treated cultures is blocked by the cysteinamine. This inhibition of the mitotic activity is followed by a qualitative and quantitative increase of nuclei per cell. Longterm cultures treated with cysteinamine survived irradiation with 700 r X rays (FIGURES 5 and 6).

Some experiments on thymine and on DNA solutions have been added to the biological experiments:

Irradiation of a thymine solution causes, as already known,[11] a decrease of UV absorption. This fact was used to test our compounds for their radioprotective capacities in a system that was independent of the physiological reactions of an organism. Our thymine solution had a concentration of 10γ/ml. in an isotonic phosphate buffer. The protecting agent was added in a concentration of 0.5 μM/ml. The final counts followed the formula

$$E = \frac{b - c}{a - c} \cdot 100 \text{ per cent},$$

a being the decrease of absorption of thymine, b the decrease of the absorption of thymine plus compound, and c the decrease of absorption of the compound

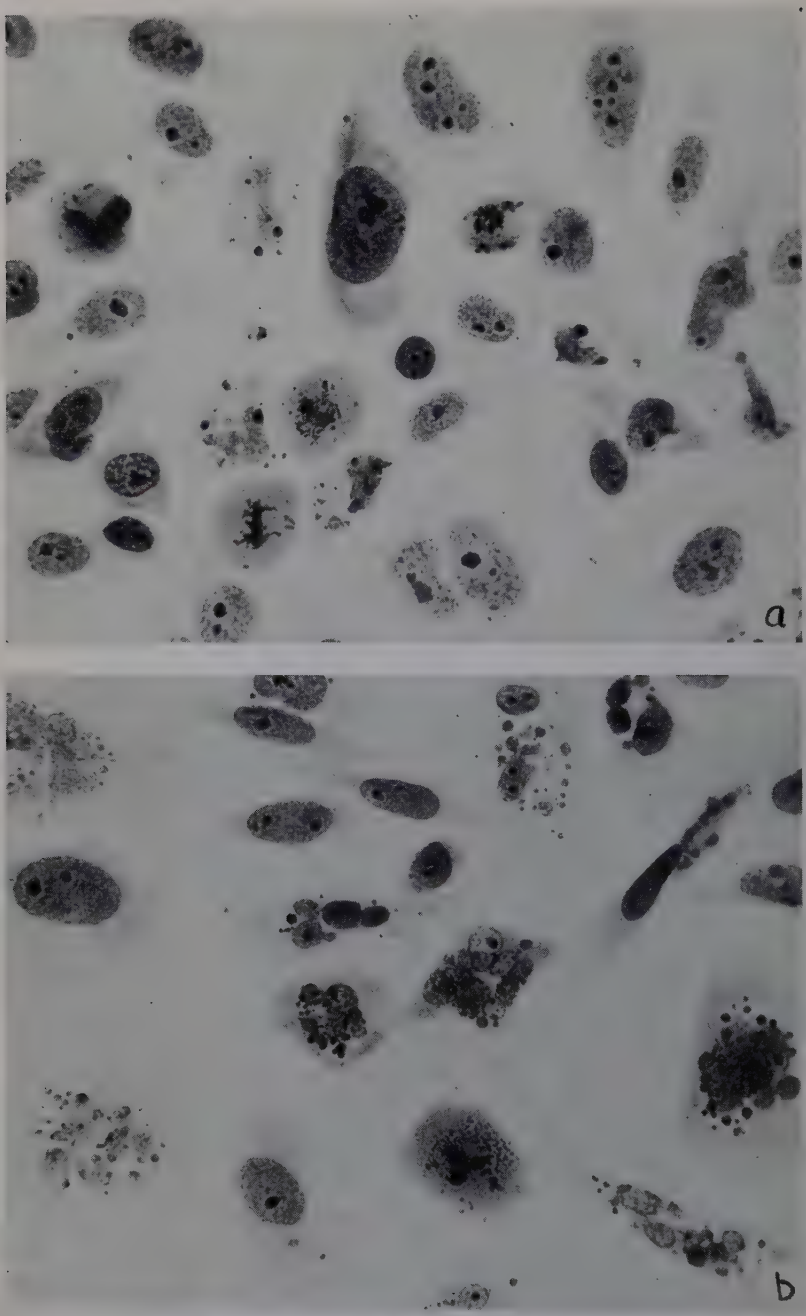
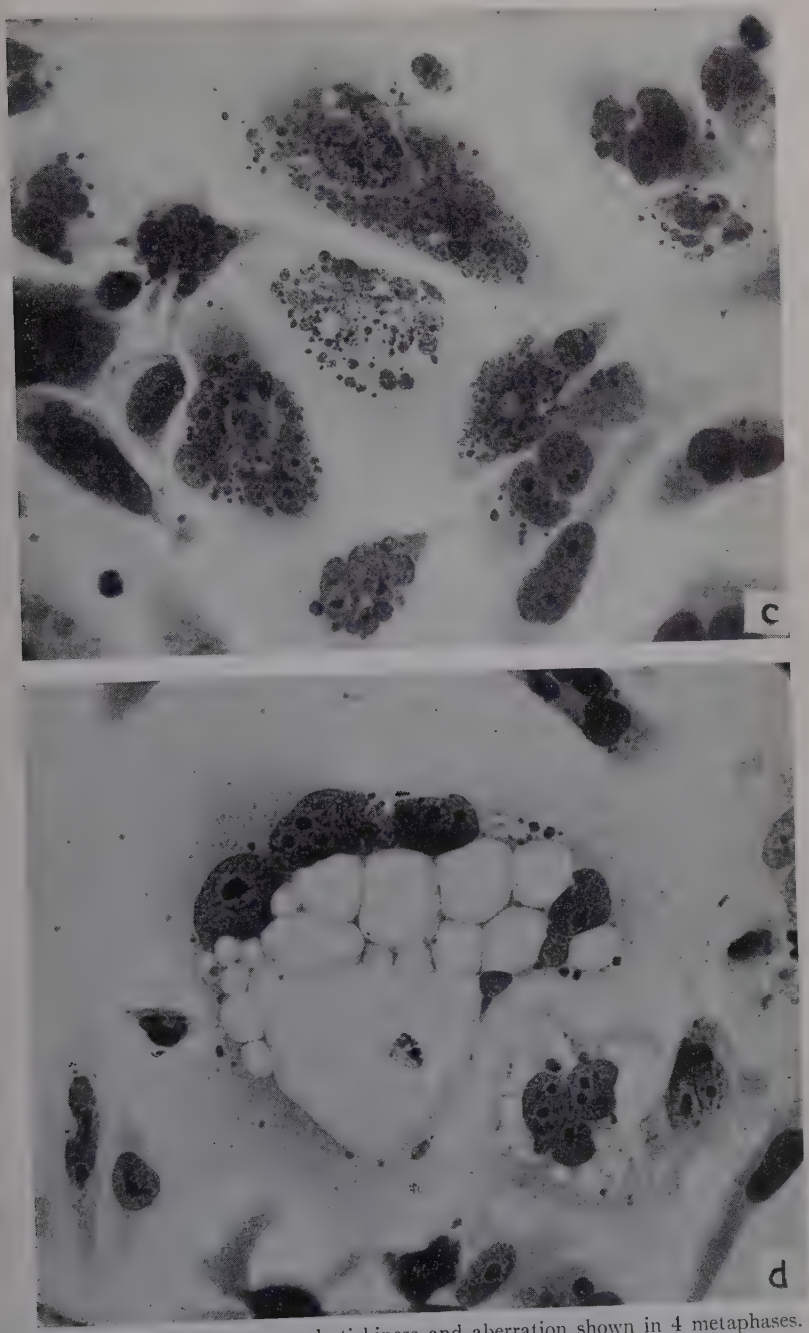


FIGURE 2. Human fibrosarcoma strain AFi. Some irradiation effects following 700-r
tended bridge between 2 daughter cells. Cytoplasm and nuclei moderately swollen. (*b*)
(*c*) Day 8: group of giant cells, most of them showing many micronuclei. Cell edema. (*d*)
Mayer's hemalum. ×420.



X rays. (*a*) Day 3: chromosomal stickiness and aberration shown in 4 metaphases. Ex-
Day 5: injured tripolar metaphase, increasing number of cells with nuclear fragmentation.
Day 11: heavily vacuolated cells. Nuclei of upper cell pressed against the cell membrane.

only. The decrease in absorption of the thymine was set equivalent to 100 per cent. The irradiation was done with the Siemens Dermopan 50 kv., at 25 mAmp., aluminum filter 1.0 mm., with the solution directly against the Roentgen-tube. The exposure was 15 min. at a rate of 2900 rpm.

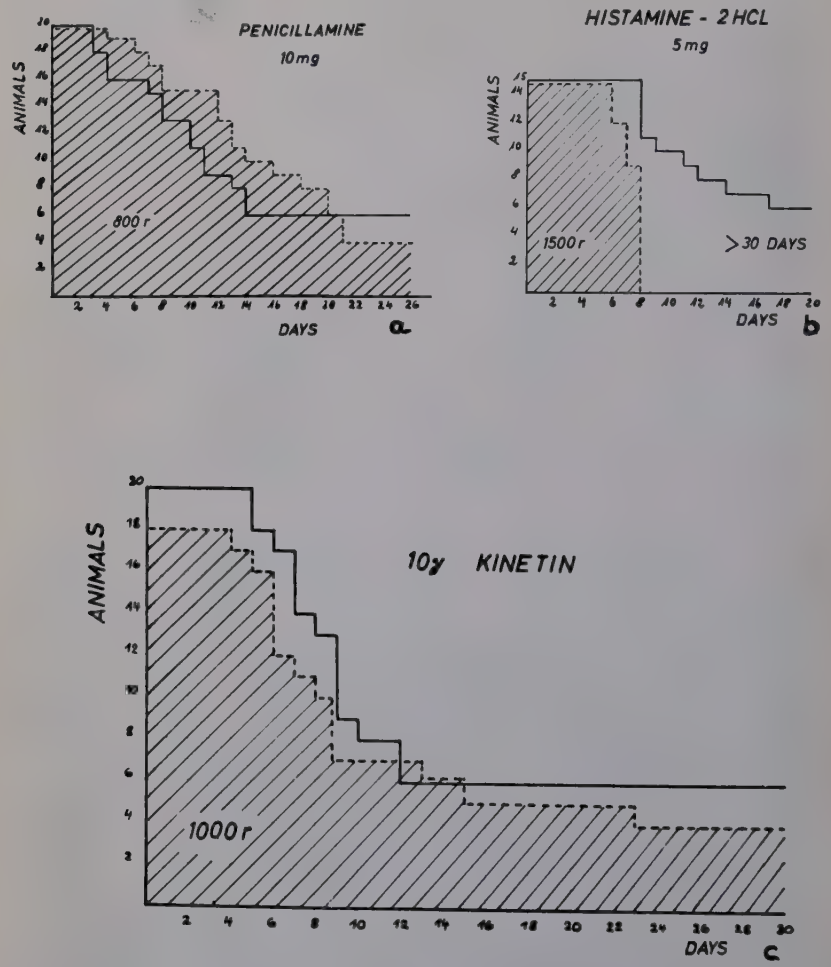


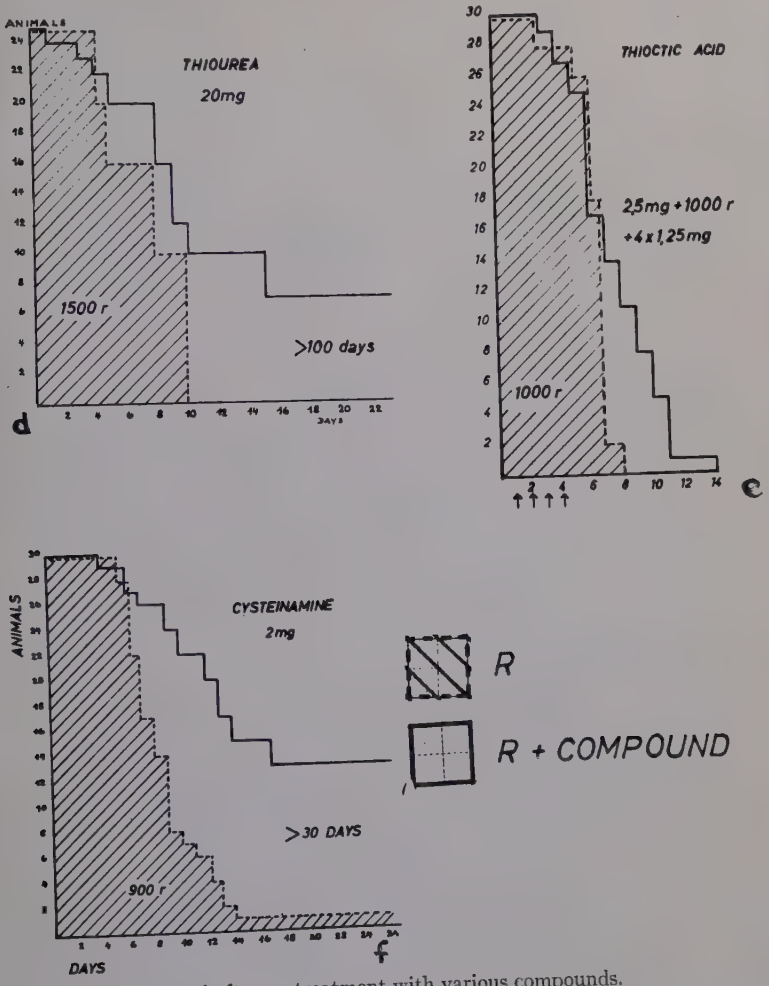
FIGURE 3. Survival curves of experimental groups of white mice

We have also studied radiation effects in DNA solutions, using the decrease of viscosity after irradiation as a criterion for protective effects. The DNS solution had a concentration of 0.05 per cent DNS in 0.2 per cent Na HCO_3. The following formula was used for evaluation:

$$E = \frac{lg\, t_0' - lg\, t_{15}'}{lg\, t_0 - lg\, t_{15}} \cdot 100 \text{ per cent},$$

In this formula t = time difference of outflow Ostwald viscosimeter; t_0 = DNA − H_2O; t_0' = (DNA plus compound) − H_2O; t_{15} = DNA irradiated minus H_2O; t_{15}' = (DNA plus compound) irradiation − H_2O.

The test substance was added at 0.5 μM/ml. Irradiation was performed



after irradiation only and after pretreatment with various compounds.

again with the Siemens Dermopan. The data are the same as with thymine except that the target distance was now 10 cm. The exposure was 15 min. at 460 rpm. Both solutions were magnetically stirred during irradiation.

The decrease of the relative viscosity in the DNA solution following irradiation is equivalent to 100 per cent irradiation effect. TABLE 1 gives some of these results. In thymine we have best protection with thiourea and tryptamine. Next is penicillamine which showed no protection in the biological experiments. Cysteine is rather protective in both experiments, how-

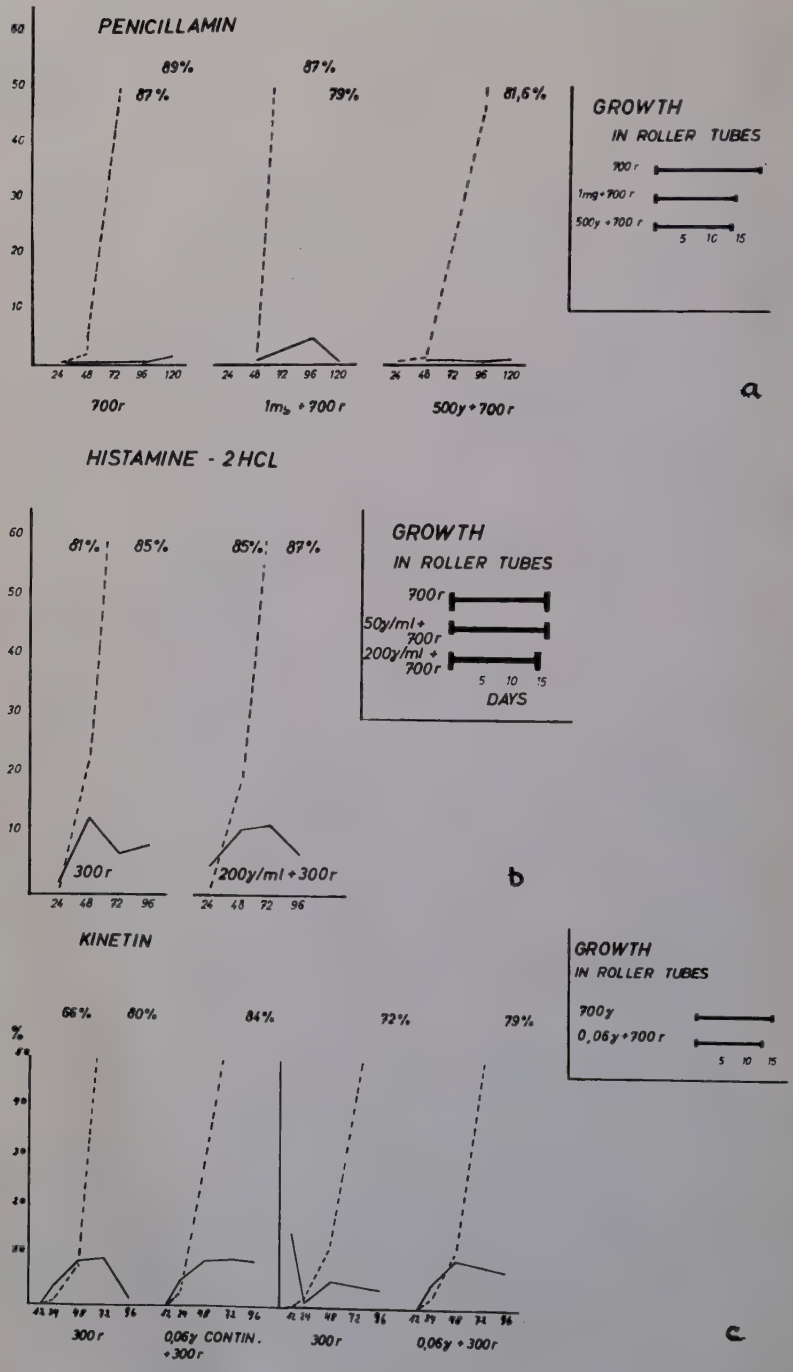
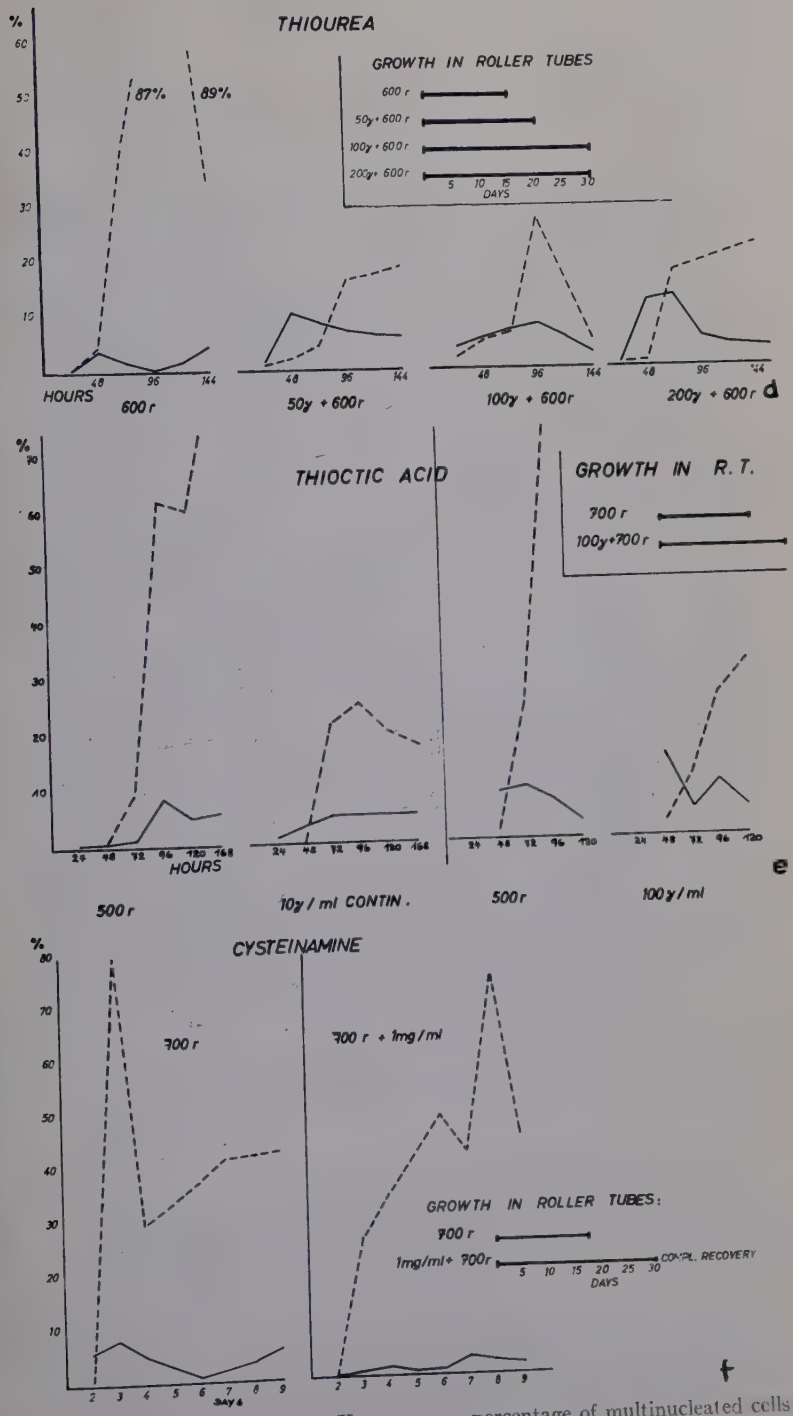


FIGURE 4. Radiation response curves of cells of the human fibrosarcoma AFi after treat-
percentage of mitoses.



ment with the various compounds. Key: – – – –, percentage of multinucleated cells; ——,

ever not as much as the thiourea. Acetic acid, KCN, and Na_2SO_4 show poor results in thymine, as well as in DNA.

TABLE 2 gives the results *in vitro* and *in vivo* and in the chemically defined solutions. Not every compound that gives some protection in thymine or DNA will do so in the animal or tissue culture. In the isolated cell we found

FIGURE 5. Living culture of strain AFi in roller tube. Cell colony 3 weeks following irradiation with 700 r. Central portion filled with dense, rounded cells. In the zone of outgrowth formation of giant cells with all sizes of vacuoles. Some moderately swollen, rounded cells, no chromosomal material detectable. Between degenerating cells a few living resting cells. ×225.

positive results only with cysteine and cysteinamine, whereas the animal reacted to other compounds. Thus far we have no definite explanation to account for this difference. In the case of the thioctic acid the low *p*H may play a role since the tissue culture cannot survive below a *p*H of 6.0.

Summary

We attempted to compare responses of mice and of a strain of human fibrosarcoma in a general way following irradiation and with an attempt to detect

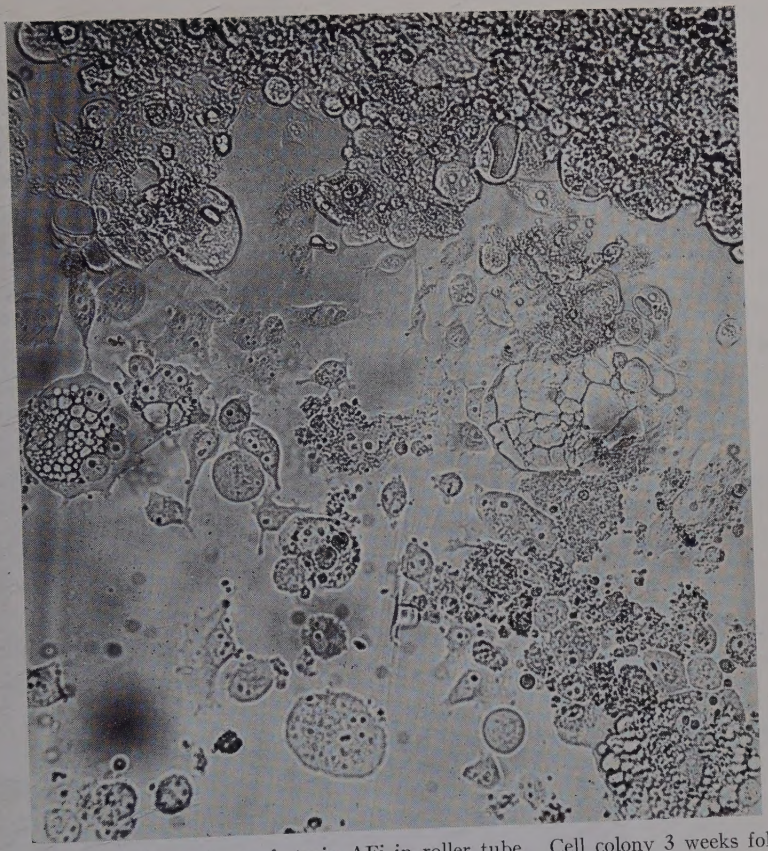


FIGURE 6. Living culture of strain AFi in roller tube. Cell colony 3 weeks following irradiation with 700 r after pretreatment with 1 mg./ml. cysteinamine. A comparison with FIGURE 5 shows the difference between the pretreated culture and the control culture that received irradiation alone. Several healthy-looking mitotic figures appear on a background of resting cells, which still show signs of injury, especially of multinucleation; however, there is no doubt of a definite survival. ×225.

TABLE 1

	Radiation effect %	
	Thymine	DNA
Histamine	19	33.5
Tryptamine	16.5	44
Thiourea	15.5	25.5
Cysteine	30	37
Cystine	35	62
Cysteinamine	24.5	31
Penicillamine	23.5	36
Thioctic acid	34	53
Kinetin	—	46
Formic acid	34	65
Acetic acid	90	98
KCN	89	66
Na_2SO_4	97	100

TABLE 2

RADIOPROTECTIVE EFFECTS OF VARIOUS COMPOUNDS WHEN TESTED IN TISSUE CULTURE IN WHITE MICE IN A THYMINE SOLUTION AND IN A DNA SOLUTION

	TC	Animal	Thymine	DNA
Histamine	−	++	+++	++
Penicillamine	−	−	++	++
Kinetin	−	−		+
Thioctic acid	∓	±	++	+
Thiourea	−	++	+++	++
Cysteine	+++	+	++	++
Cysteinamine	+++	+++	++	++

protection with a series of compounds. The best results have been obtained with cysteine and cysteinamine. The great variety of our objects gave us results on a broad spectrum. It is not our intention to try to compare these results beyond a certain level; however we hope to be able to add some fragments to the big mosaic of experiments on radioprotection.

Acknowledgment

I am indebted to A. Mayer for his cooperation and to L. Döring for her technical assistance.

References

1. VAN BEKKUM, D. W. & J. DE GROOT. 1956. Observations on chemical protection *in vivo* and *in vitro*. *In* Progress in Radiobiology. J. S. Mitchell and B. E. Holmes, Eds. Oliver and Boyd. London, England.
2. SCHLEICH, A., M. K. GEY & G. O. GEY. 1961. Attempts at influencing the radiation response of a human tumor strain AFi and HeLa in continuous cultures: a test of Synkavit (synthetic Vitamin K) as radiosensitizer. Ann. N.Y. Acad. Sci. **95** (2) : 774.
3. MILLER, C. O., F. SKOOG, F. S. OKUMURA, M. H. VON SALTZA & F. M. STRONG. 1956. Isolation, structure, and synthesis of kinetin, a substance promoting cell division. J. Am. Chem. Soc. **78:** 1375.
4. GUTTMAN, R. & S. W. BROWN. 1959. The influence of indole acetic acid and kinetin on radiation damage to dividing root meristems. The Nucleus. **2:** 111.
5. LETTRÉ, H., H. BALLWEG, H. ENDO, A. SCHLEICH & W. SIEBS. 1958. Some studies with analogues of kinetin. Biochem. Pharm. **1:** 137–140. Pergamon Press Ltd. London, England.
6. KOFLER, E., G. BALDINI & E. BALDOLI. 1957. Primi dati sull' azione prottetiva dell' acido tiocto di fronte al raggi roentgen. Boll. soc. ital. biol. sper. **33**(4)**:** 408.
7. CHÈVREMONT-CONHAIRE, S. & M. CHÈVREMONT. 1953. Action de la β-mercaptoéthylamine sur la croissance et la mitose en culture de tissus. Compt. rend. de l'Association Anat. XL. Réunion. Bordeaux, France.
8. TRABERT-VAN DER MAESEN, M. 1957. Etude comparative de la croissance et la mitose dans des cultures irradiées avec ou sans traitement préalable à la cystéamine. Compt. rend. soc. biol. 28. Vol. CLI **8–9:** 1624.
9. OFTEDAL, P., R. OFTEBRO & R. EKER. 1958. Radioprotective properties of cystamine, cysteamine cysteine when tested with chick fibroblast *in vitro*. Nature. **181:** 344.
10. THERKELSEN, A. J. 1960. Biological actions of cysteamine. Toxicity and protection against Roentgen rays and nitrogen mustard. Dissertation. The Institute of General Pathology. University of Aarhus.
11. SCHOLES, G. & J. WEISS. 1952. Chemical actions of X rays on nucleic acids and related substances in aqueous systems. Exptl. Cell Research. : 219.

Printed in the United States of America

MONOGRAPHIC PUBLICATIONS

OF

THE NEW YORK ACADEMY OF SCIENCES

(Lyceum of Natural History, 1817–1876)

(1) The Annals (octavo series), established in 1823, contain the scientific contributions and reports of researches, together with the records of meetings of the Academy. The articles that comprise each volume are printed separately, each in its own cover, and are distributed immediately upon publication. The price of the separate articles depends upon their length and the number of illustrations, and may be ascertained upon application to the Executive Director of the Academy.

Current numbers of the Annals are sent free to all Members of the Academy desiring them.

(2) The Special Publications, established in 1939, are issued at irregular intervals as cloth-bound volumes. The price of each volume will be advertised at time of issue.

(3) The Memoirs (quarto series), established in 1895, are issued at irregular intervals. It is intended that each volume shall be devoted to monographs relating to some particular department of science. Volume I, Part 1 is devoted to Astronomical Memoirs, Volume II to Zoological Memoirs. No more parts of the Memoirs have been published to date. The price is one dollar per part.

(4) The Scientific Survey of Porto Rico and the Virgin Islands (octavo series), established in 1919, gives the detailed reports of the anthropological, botanical, geological, paleontological, zoological, and meteorological surveys of these islands.

Subscriptions and inquiries concerning current and back numbers of any of the publications of the Academy should be addressed to

EXECUTIVE DIRECTOR
The New York Academy of Sciences
2 East Sixty-third Street
New York 21, N. Y.